A 3-dimensional endometrial organotypic model simulating the acute inflammatory decidualisation initiation phase with epithelial induction of the key endometrial receptivity marker, integrin V3

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A 3-dimensional endometrial organotypic model simulating the acute inflammatory decidualisation initiation phase with epithelial induction of the key endometrial receptivity marker, integrin αVβ3

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We thank the reviewers, Associate Editor and Editor-in-Chief for their further evaluation and comments. We have prepared a revised manuscript based on the feedback, and provide a point-by-point response to the comments below. The additions and sections linked to the comments are shown in red font within our revised manuscript, and the changed wording throughout the manuscript (as suggested by the Associate Editor), indicated in light blue font.

Reviewer: 2

Comments to the Author:

Although the reported findings are quite interesting, they are also quite limited. Using a single molecule for assessment of decidualization and another single molecule for assessment of receptivity is a superficial analysis, despite the chosen markers being very good ones. In fact, the single measure of decidualization, IGFBP-1 immunofluorescence, remains unquantified. The representative images (figure 1) for IGFBP-1 would seem quite convincing, but there is no measure of variability using quantitative methods. The alphavbeta3 expression is quantified, but exhibits only a 3-fold increase. This is surprising, as the in vivo situation shows very little epithelial staining without stimulation and quite robust staining afterwards (except for endothelium). Thus, the markers are good ones, but without other markers, the reader is left to guess whether this truly recapitulates endometrial functional changes due to hormonal decidualization stimulus.

In addition to IGFBP-1 being a robust marker for assessing differentiation status and quality of the decidual response of ESCs in culture, we also observed clear morphological changes upon hormonal stimulation (such as enlargement and rounding of the nucleus, and larger cell size) in ESCs upon hormonal stimulation (Figure 1A), which also induced a transient upregulation of ST2L, on day 6 (Figure 1B), indicating an acute inflammatory decidual response (lines 89-90, 92, and 291-298). Decidualising ESCs induce a transitory, acute autoinflammatory response, through secretion of IL-33 (a key regulator of the innate immune response), whilst upregulating the expression of its receptor, ST2L, and this signalling induces receptivity gene expression in the overlying epithelium (lines 401-405), which was indicated by the induction of epithelial $\alpha V\beta 3$.

We acknowledge, however, that further validation would be beneficial to confirm an accurate representation of these key endometrial functional changes in our model, and we have added a section within our manuscript to describe the limitations of our study, which could include, for example, the iGenomix® Endometrial Receptivity Array (ERA) genomic tool (lines 511-512).

In this regard, changes in steroid hormone receptors after decidualization stimulus would enlightening. The response that steroid receptors can be variable due to hormonal effects, and thus were not evaluated, is quite concerning. Eutherian mammals, including humans, universally show down regulation of epithelial ER and PR prior to implantation. Thus, demonstration of epithelial ER and/or PR

changes after decidual stimulus would be an important validation of the model and further evidence that the response to E+P+cAMP is mediated by the E+P stimulus.

While the lack of induction of epithelial integrin αVβ3 (upon hormonal stimulation) by EECs in monoculture suggests that the induction of epithelial αVβ3 in our co-culture model may have resulted from EEC-ESC crosstalk, following potential hormonal downregulation of epithelial PR and ER, further experiments would be needed to confirm this (lines 478-483). The '*Limitations of the study*' section therefore now states that characterisation of EEC and ESC steroid receptor expression upon hormonal stimulation could be conducted for further validation of a receptive endometrial phenotype (lines 510-511).

The response about glandular versus luminal epithelium is confusing. There are molecular markers that have been proposed by DeMayo, Spencer and others, e.g. FOXA2 for glandular. Of course, there are light microscopic and ultrastructural markers as well. If none of those were done, it is unclear why the authors state that alphaVbeta3 appears on both luminal and glandular epithelium, but then indicate that it was luminal phenotype because they saw alphavbeta3 expression. This is a very minor issue.

We apologise for the lack of clarity that led to confusion. We have discussed luminal epithelial αVβ3 expression throughout the manuscript, as this is perceived as the fundamental site for endometrial receptivity (line 443). The comment regarding αVβ3 expression in the glandular epithelium was in response to a question in the previous critique, regarding differential receptor expression patterns in luminal versus glandular epithelium, and we stated that αVβ3 appears on both luminal and glandular epithelium.

We have deleted the reference to glandular epithelial αVβ3 expression from the manuscript, and we did not observe any gland-like structures within the epithelial monolayer in our 3D model.

As mentioned in the previous critique, there is no photomicrograph evidence that the structure represents a 3-D model of the endometrium, such as seen in figure 3A. The studies cited in response to the critique do not use the combination of cell types used in this study, thus it cannot be claimed that such recapitulation is "well-established". Cell migration can easily occur in 3-D models and an intact epithelium would be necessary for a true model of receptivity that the title suggests - one that could measure function.

The '*Limitations of the study*' section, which incorporates suggestions of how our model can benefit from additional confirmation of endometrial receptivity, now includes a statement that photomicrographic verification of accurately representative 3D spatial relationships of the cell-types would further validate our model (lines 509-510).

Reviewer: 1

Comments to the Author:

In Supplement figure 1B, they authors show that integrin αVβ3 expression by endometrial epithelial cells (EECs) alone did not change following hormone stimulation. But were the EECs grown in monolayer 2D or 3D culture with Matrigel layer when they did this experiment? This needs to be clarified since some marker proteins may have different expression patterns 2D cultures compared to 3D cultures.

They were grown in monolayer, on Matrigel-coated plates. However, while there may be discrepancies in protein expression associated with degree of Matrigel thickness and in 2D vs. 3D culture models (although, even in our 3D construct, the EECs were seeded in a 2D monolayer above the 3D ESC assembly), rising progesterone levels are known to downregulate endometrial epithelial PR and ER expression, and therefore the hormonal stimulation is likely to not act directly on EECs to induce αVβ3 expression, although we concede that further investigation would be needed to confirm this (lines 478-483).

[*If the editorial team deems it necessary, the following can be added to the manuscript at line 478: '…compartment. While there may be a degree of differential protein expression patterns associated with Matrigel thickness and in 2D vs. 3D cell culture (Liu, Qi et al. 2018, Edmondson, Broglie et al. 2014), rising progesterone levels…']

Associate Editor's comment to Author:

The two reviewers have given quite disparate opinions on the resubmitted manuscript. The overall criticism from R2 is mainly that your suggested model is not validated for functionality, and no firm conclusions can be drawn. However, the model can be seen as a new concept to study receptivity, but in need for further development and validation. As such it can be of interest to the readers of HRO.

I have the following suggestions:

1. Change the title and the wording throughout the manuscript to express that the model has a potential to improve receptivity insights. In the title and in the Summary answer in the abstract, 'novel' could be exchanged with 'potential'. In Wider implications in the Abstract, 'offers' can be replaced by 'may offer' etc. to highlight that the model needs further validation.

As suggested, we have now removed the word 'novel' from both the title and the Abstract. We have also changed the title to: **A 3-dimensional endometrial organotypic model simulating the acute inflammatory decidualisation initiation phase with epithelial induction of the key endometrial receptivity marker, integrin αVβ3**

We have changed the summary answer in the Abstract to: We present a new concept to investigate endometrial receptivity, with a 3-dimensional (3D) organotypic model to simulate an early and transient acute autoinflammatory decidual status that resolves in the induction of a receptive endometrial phenotype.
We believe these changes better represent our model as a new concept to investigate endometrial receptivity, via a simplified simulation of the uterine microenvironment leading up to the induction of a receptive endometrial phenotype, rather than stating that we present 'a novel *in vitro* 3-dimensional (3D) organotypic functional co-culture model representing a receptive endometrial phenotype' (as in the previous version of the manuscript).

As suggested, we have changed the wording throughout (light blue font).

2. The Discussion section could be more structured. Under a subheading Limitations, the points raised by Reviewer 2 could be summarized, to emphasize that the model can be further developed. A specific question from Reviewer 1 regarding Suppl Fig 1B needs to be clarified. Also, line 160, the number of patients (three) could be added; "obtained from three women".

We have restructured the Discussion and added the following: '*Limitations of the study*' and '*Concluding remarks*'.

We have included the points raised by Reviewer 2 in the *Limitations of the study*' section, and we have addressed the specific question from Reviewer 1 in lines 478-483.

We have also changed the wording to "obtained from three women" (now line 177).

Editor-in-Chief
Comments to the Authors:
The views of the AE and one of the reviewers indicate the need to address a number of residual issues before this paper can be considered suitable for acceptance by HROpen. This paper does need a "What does this mean for Patients" lay summary.

We have now added a '*What does this mean for patients?'* lay summary.

The authors have one more opportunity to respond to the queries and comply with the suggestions from the AE.

We thank the Editor-in-Chief for this chance to respond to the queries, which we have now addressed,

and we have made changes to our manuscript in order to comply with the Associate Editor's suggestions.

In addition, they need to explain what they mean by "endometrial factor infertility" in line 493 as this is not a term used in clinical practice.

We were referring to abnormalities of the endometrium being the cause of infertility as 'endometrial factor infertility', as we have come across this term being used in a few papers (albeit without proper descriptions of its meaning). However, we realise that this does not need to be additionally stated, since we have described the dysregulation of endometrial events (such as decidualisation and induction of endometrial receptivity) being implicated in infertility, making the statement superfluous. We have therefore removed this redundant point.

A 3-dimensional endometrial organotypic model simulating the acute inflammatory decidualisation initiation phase with epithelial induction of the key endometrial receptivity marker, integrin αVβ3

Running title: 3D *in vitro* receptive endometrium

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Abstract

Study question: There is a distinct lack of directly relevant *in vitro* models of endometrial receptivity; is it possible to develop a simplified physiological *in vitro* system representing the key cell-types associated with a receptive endometrial phenotype?

Summary answer: We present a new concept to investigate endometrial receptivity, with a 3-dimensional (3D) organotypic co-culture model to simulate an early and transient acute autoinflammatory decidual status that resolves in the induction of a receptive endometrial phenotype.

What is known already: Embryo implantation is dependent on a receptive uterine environment. Ovarian steroids drive post-ovulation structural and functional changes in the endometrium, which becomes transiently receptive for an implanting conceptus, termed the 'window of implantation', and dysregulation of endometrial receptivity is implicated in a range of reproductive, obstetric, and gynaecological disorders and malignancies. The interactions that take place within the uterine microenvironment during this time are not fully understood, and human studies are constrained by a lack of access to uterine tissue from specific time-points during the menstrual cycle. Physiologically relevant *in vitro* model systems are therefore fundamental for conducting investigations to better understand the cellular and molecular mechanisms controlling endometrial receptivity.

Participants/materials, setting, methods: An endometrial stromal cell (ESC) line, and endometrial epithelial cells (EECs) isolated from uterine biopsy tissue and expanded *in vitro* by conditional reprogramming, were used throughout the study. Immunocytochemical and flow cytometric analyses were used to confirm epithelial phenotype following conditional reprogramming of EECs. To construct an endometrial organotypic co-culture model, ESCs were embedded within a 3D growth factor-reduced Matrigel structure, with a single layer of conditionally reprogrammed EECs seeded on top. Cells were stimulated with increasing doses of medroxyprogesterone acetate, cyclic adenosine monophosphate and estradiol, in order to induce ESC decidual transformation and endometrial receptivity. Decidual response and the induction of a receptive epithelial phenotype were assessed by immunocytochemical detection and quantitative in-cell western® analyses, respectively.

Main results and the role of chance: A transient upregulation of the IL-33 receptor, ST2L, was observed in ESCs, indicating a transient autoinflammatory decidual response to the hormonal stimulation, known to induce receptivity gene expression in the overlying epithelium. Hormonal stimulation induced the EEC expression of the key marker of endometrial receptivity, integrin αVβ3

(n = 8; *P <0.05; ***P < 0.0001). To our knowledge, this is the first demonstration of a dedicated endometrial organotypic model, that has been developed to investigate endometrial receptivity, via the recapitulation of an early decidual transitory acute autoinflammatory phase and induction of an epithelial phenotypic change, to represent a receptive endometrial status.

Limitations, reasons for caution: This simplified *in vitro* ESC-EEC co-culture system may be only partly representative of more complex *in vivo* conditions.

Wider implications of the findings: The 3D endometrial organotypic model presented here may offer a valuable tool for investigating a range of reproductive, obstetric, and gynaecological disorders, to improve outcomes for assisted reproductive technologies, and for the development of advances in contraceptive methods.

Study funding/competing interest(s): This work was supported in part by an MRC Centre Grant (project reference MR/N022556/1). RF was the recipient of a Moray Endowment award and a Barbour Watson Trust award. C-JL is a Royal Society of Edinburgh Personal Research Fellow, funded by the Scottish Government. The authors have no conflicts of interest to declare.

Keywords: endometrial receptivity; window of implantation; decidualisation; endometrial organotypic model; conditional reprogramming

What does this mean for patients?

During the first stage of the menstrual cycle, estrogen secretion from the developing ovarian follicle promotes growth of the endometrium (the lining of the womb). Following ovulation, rising progesterone and estrogen levels, produced by the corpus luteum (the remaining structure of the ovarian follicle that contained the maturing egg before its release during ovulation), promote structural and functional changes in the endometrium, in preparation for the 'window of implantation' – a period of 2–5 days when the endometrium is optimally receptive to an implanting embryo. This period of optimal endometrial receptivity is not only crucial for successful embryo implantation, but abnormal molecular and cellular events in the endometrium during this transient period have been implicated in fertility problems, obstetric complications, gynaecological disorders, and endometrial cancer. In this study, we have developed a simplified cellular model, with physiologically appropriate hormonal stimulation, to investigate endometrial receptivity. A more comprehensive understanding of these events can lead to the development of new interventions to promote pregnancy success, long-term maternal and fetal health, women's health, as well as for improving contraceptive methods, and this new concept may be able to aid investigations to better understand the complex mechanisms involved in the generation of endometrial receptivity.

Introduction

Embryo implantation is a critical event in human pregnancy that is reliant on a receptive uterine environment. The cycling endometrium undergoes profound changes in women, leading to a carefully timed and defined period during which an embryo is able to attach and invade into a receptive uterus, resulting in the establishment of a successful pregnancy (Norwitz, Schust et al. 2001). Ovarian steroids, estrogen and progesterone, drive structural and functional changes in the uterine lining, preparing it for the implantation of a conceptus. The uterine lining, known as the endometrium, consists of a fibroblast-like stromal matrix lined by a single layer of columnar epithelium. Following ovulation, dynamic changes take place in the endometrial stromal cell (ESC) morphology, which undergo mesenchymal-to-epithelial transformation, and begin to differentiate into large, secretory, 'decidualised' stromal cells, in response to rising progesterone levels produced by the corpus luteum (Gellersen, Brosens et al. 2007, Salamonsen, Nie et al. 2009). Decidual transformation of ESCs is associated with enlargement and rounding of the nucleus, increased number of nucleoli, rough endoplasmic reticulum and Golgi complex expansion, and accumulation of glycogen and lipid droplets in the expanding cytoplasm (Gellersen and Brosens 2014, Kajihara, Tanaka et al. 2014, Okada, Tsuzuki et al. 2018).

Decidualisation is a dynamic, multistep progression of events, comprising 3 critical transitory phases: (i) an acute inflammatory initiation phase that subsequently transitions to (ii) an anti-inflammatory secretory phase during which time embryo implantation takes place, followed by (iii) a final resolution phase (Gellersen and Brosens 2014). First, ESCs undergo cell cycle exit at G_0/G_1 and mount a transient pro-inflammatory response generated by a self-limiting autoinflammatory response, which, in turn, results in the expression of key receptivity genes in the overlying endometrial surface luminal epithelium (Salker, Nautiyal et al. 2012). This renders the endometrium receptive for embryo implantation for a limited period of time: the 'window of implantation'. This period of optimal endometrial receptivity begins approximately 6 days post-ovulation and lasts 2–5 days (i.e. approximately between days 20 and 25 of an idealised 28-day cycle) (Denker 1993). A receptive endometrial phenotype is not only imperative for embryo implantation and pregnancy success, but aberrant decidual transformation and dysregulation of uterine receptivity have also been implicated in several obstetric complications, gynaecological disorders and cancer (Norwitz 2006, Strowitzki, Germeyer et al. 2006, Cartwright, Fraser et al. 2010, Lessey 2011, Patel and Lessey 2011, Gellersen and Brosens 2014, Timeva, Shterev et al. 2014, Rabaglino, Post Uiterweer et al. 2015, Tan, Hang et al. 2015, Conrad, Rabaglino et al. 2017).

Interactions between different cell-types have reciprocal effects on cell phenotypes and ensuing functions (Freshney 2005). The same is true for uterine compartments and the contributions of these interactions to endometrial receptivity, since decidual transformation of the stroma confers its ability to create paracrine gradients necessary for expression of evolutionarily conserved molecules by the luminal epithelium that are fundamental for embryo implantation (Achache and Revel 2006, Salker, Nautiyal et al. 2012). It has been demonstrated that endometrial receptivity is mediated by the activation of autoregulatory feedback loops in decidualising ESCs underlying the luminal epithelium, which activate the sequential expression of pro- and anti-inflammatory gene networks, and that ESCs can exert this function independent of local immune cells (Salker, Nautiyal et al. 2012). As such, it is evident that there is a co-dependent relationship between the endometrial stroma and epithelium, to prepare the uterus for pregnancy (Cakmak and Taylor 2011), with ESC decidual transformation being a prerequisite for the generation of endometrial receptivity (Vinketova, Mourdjeva et al. 2016, Yu, Berga et al. 2016). The current study focuses on the uterine phenotype during the acute inflammatory initiation phase of decidual transformation implicated in the generation of endometrial receptivity.

The mechanisms that control decidualisation and endometrial receptivity are highly complex, and we do not yet fully understand all the interactions that take place within the uterine microenvironment during this time. Uterine competence for embryo implantation sets the foundation for a successful pregnancy pathway; it is only when a clearer picture of the relative contributions of the cellular and molecular mechanisms leading to a receptive endometrial status become apparent, that the pathophysiology of several reproductive, obstetric and gynaecological disorders can be further defined, and appropriate interventions can be developed to promote pregnancy success as well as long-term maternal and fetal health. Likewise, a better understanding of these mechanisms will also be beneficial for innovations in contraceptive methods. We have developed a simplified 3-dimensional (3D) endometrial organotypic model to investigate endometrial receptivity, in which we simulate an early acute inflammatory endometrial status, that resolves in the generation of a receptive luminal epithelial phenotype, known as the 'window of implantation'. Organotypic culture refers to *in vitro* cell culture models in which two or more previously disaggregated cell-types are recombined in experimentally determined ratios and spatial relationships to reconstruct a constituent of the corresponding *in vivo* organ, as opposed to histiotypic cultures (high density culture of a single cell-type within a 3D matrix) or organoid cultures

(simplified, self-organising stem cell-derived 3D multicellular aggregates with the ability to mimic its *in vivo* organ counterpart) (Freshney 2005, Simian and Bissell 2017). An endometrial organotypic culture model, albeit a simplified representation, is able to better recapitulate the morphological and functional features of the *in vivo* uterine microenvironment, than is possible conventional 2-dimensional or even histiotypic cell culture methods. Physiologically representative *in vitro* model systems are vital for investigating the mechanisms implicated in endometrial receptivity, due to the ethical and logistical limitations of human studies. While there are reports of several *in vitro* endometrial co-culture models in the literature (Bentin-Ley, Horn et al. 2000, Arnold, Kaufman et al. 2001, Bläuer, Heinonen et al. 2005, Wang, Pilla et al. 2012, Chen, Erikson et al. 2013), these are not representative of the early acute inflammatory decidualisation phase that gives rise to a transiently receptive epithelial phenotype. The new functional co-culture system presented here may offer a convenient and accessible tool to improve our comprehension of interactions in the uterine microenvironment during this transitory phase.

Materials and methods

Culture and hormonal stimulation of the endometrial stromal cell line St-T1b a

The human ESC-derived telomerase-immortalised cell line, St-T1b (Samalecos, Reimann et al. 2009), kindly provided by Professor Jan Brosens (University of Warwick, UK), was maintained in phenol red-free Dulbecco's modified Eagle medium DMEM/Ham's F12 (DMEM/F12; Invitrogen, Renfrew, UK) with 10% steroid-depleted fetal calf serum (FCS) supplemented with 2mM L-glutamine, 1 µg/ml insulin, 0.3 ng/ml 17β-estradiol (E2), 50 µg/ml penicillin, 50 µg/ml streptomycin, and 0.2% Primocin (Invivogen, Toulouse, France) (ESC medium) at 37°C in an atmosphere of 5% CO_2. Phenol red-free medium was used in all experiments, due to phenol red's known estrogenic activity (Berthois, Katzenellenbogen et al. 1986). To induce decidualisation, cells were treated with minimal medium 1 (MM1; ESC medium without insulin and E2) containing increasing concentrations of the progestin, medroxyprogesterone acetate (MPA), 8-bromoadenosine 3',5'-cyclic adenosine monophosphate (8-Br-cAMP; Cambridge Bioscience, Cambridge, UK) and E2, or MM1 with 0.001% ethanol (EtOH) (Table 1) every 48 hours, and cultured over 8 days at 37°C in an atmosphere of 5% CO_2. All reagents for St-T1b cell culture were purchased from Sigma-Aldrich (Sigma-Aldrich, Dorset UK) unless stated otherwise. Cultured cells were fixed in 4% paraformaldehyde (PFA) on days 4, 6 and 8 for immunocytochemical analysis.

Isolation of epithelial cells from endometrial biopsies and ethical approval

Primary human endometrial biopsy tissue was obtained from three women attending gynaecological outpatient departments in NHS Lothian. Written informed consent was obtained from participants and ethical approval granted from Lothian Research Ethics Committee (REC 16/ES/0007). The women reported regular menstrual cycles and did not have exogenous hormone exposure for 2 months prior to biopsy. Women receiving hormonal therapy, suffering from endometriosis or those with fibroids of >3 cm were excluded. Human endometrial epithelial cells (EECs) were isolated from endometrial biopsies by tissue digestion and separation from ESCs. Endometrial tissue was minced using scalpel blades, followed by digestion with 100 µg/ml collagenase II and 0.25 µg/ml DNAse I (Sigma-Aldrich, Dorset UK) for 1.5 h at 37°C. The tissue homogenate was then sequentially strained through 70-µm and 40-µm membrane filters to separate glandular epithelium from ESCs. The membrane filters were back-washed with PBS to retrieve endometrial gland clumps, further rinsed with PBS to flush out any digestion medium, and mixed well to disperse clumps. EECs were then suspended in PBS and centrifuged at 500 x *g* for 5 minutes at room temperature (RT), supernatant subsequently discarded, followed by resuspension in PBS and centrifugation at 500 x *g* for 5 minutes at RT.

Expansion of endometrial epithelial cells by conditional reprogramming

EECs were rapidly expanded *in vitro* by conditional reprogramming with the use of Y-27632 (a Rho kinase inhibitor) and fibroblast feeder cells. First, 3T3 Swiss Albino fibroblasts (cell line obtained from the European Collection of Authenticated Cell Culture, Public Health England, Salisbury, UK) were grown in MM1 to approximately 80% confluence in T175 flasks, trypsinated, washed, resuspended in MM1 and irradiated at 30 Gy. The irradiated cells were washed, cultured at 37°C in an atmosphere of 5% CO_2, and conditioned medium collected 72 hours post-irradiation. EEC medium was prepared with phenol red-free DMEM/Ham's F12 containing 10% steroid-depleted FCS, and supplemented with 2mM L-glutamine, 5 µg/ml insulin, 24 µg/ml adenine, 0.4 µg/ml hydrocortisone, 10 ng/ml epidermal growth (EGF), 8.4 ng/ml cholera toxin, 10 µmol/l Y-27632 (Cambridge Bioscience, Cambridge, UK, 10 µg/ml gentamycin and 0.25 µg/ml amphotericin. The irradiated 3T3 conditioned medium was added to EEC medium in a 1:3 ratio, 1 part of IR 3T3 conditioned media to 3 parts of EEC medium; CREEC medium (conditional reprogramming EEC medium), and the EECs maintained in CREEC medium at 37°C in an atmosphere of 5% CO_2. This method has previously been shown to directly alter cell growth without selecting for a small sub-population of stem-like cells, while retaining a normal non-tumourigenic karyotype, and

conditionally inducing an indefinite proliferative state in primary mammalian epithelial cells (Liu, Ory et al. 2012, Suprynowicz, Upadhyay et al. 2012, Palechor-Ceron, Suprynowicz et al. 2013). All reagents for EEC culture and conditional reprogramming were purchased from Sigma-Aldrich (Sigma-Aldrich, Dorset UK) unless stated otherwise. EECs can be cryopreserved using mFreSR™ cryopreservation medium (Stemcell Technologies, Cambridge, UK). Cultured EECs were fixed in 4% PFA for immunocytochemical analysis.

Immunocytochemical confirmation of ESC decidualisation and EEC phenotype

Fixed cells (St-T1b cells and EECs) were permeabilised with 0.1% Triton X-100 in PBS for 10 minutes, and washed three times with 0.1% Tween-20 in PBS (PBST). Cells were then blocked with 5% BSA in PBS for 1 hour at RT, incubated with primary antibody overnight at 4°C, washed three times with 0.1% PBST, subsequently incubated with secondary antibody and 1:10,000 DAPI for 30 minutes at RT in the dark, followed by a final wash with PBS. Primary antibodies used were rabbit anti-human IGFBP-1 (Abcam, Cambridge, UK; ab111203; 1:100), rat anti-mouse ST2L (IL-33R/ST2) (eBioscience, Cheshire, UK; 17-9335-82; 1:100), rabbit anti-human cytokeratin-18 conjugated to phycoerythrin (Abcam, Cambridge, UK; ab218288; 1:1000), rabbit anti-human vimentin (New England Biolabs, Hitchin, UK; 5741; 1:100), and mouse anti-human integrin αVβ3 (Abcam, Cambridge, UK; ab190147; 1:100). A goat anti-rabbit antibody conjugated to Alexa Fluor 546 (Invitrogen, Renfrew, UK; A-11071; 1:300), a donkey anti-rabbit antibody conjugated to Alexa Fluor 568 (Invitrogen, Renfrew, UK; A10042; 1:250) and a donkey anti-mouse antibody conjugated to Alexa Fluor 488 (Invitrogen, Renfrew, UK; A21202; 1:500) were used as secondary antibodies. The cytokeratin-18 staining did not require incubation with a secondary antibody. The ST2L staining did not require cell permeabalisation for detection, but did require a signal amplification step after primary antibody incubation, with a biotinylated goat anti-rat antibody (Vector Laboratories, Peterborough, UK; BA-4000; 1:100) for 30 minutes at RT in the dark, followed by three washes with 0.1% PBST. Cells were then incubated with streptavidin conjugated to Alexa Fluor 488 (Invitrogen, Renfrew, UK; s11223; 1:200) and 1 mg/ml Hoechst 33342 (Invitrogen, Renfrew, UK; H3570) for 30 minutes at RT in the dark, and then washed with PBS. Imaging was conducted on an Olympus IX71 microscope with a QImaging optiMOS camera and CoolLED PE4000 light source (Olympus, Tokyo, Japan) or a Zeiss Axio Observer 7 microscope (Carl Zeiss Ltd, Cambridge, UK) with a Hamamatsu ORCA-Flash LT camera (Hamamatsu Protonics, Hertfordshire, UK) and Zeiss Colibri 7 LED light source (Carl Zeiss Ltd, Cambridge, UK). Images were analysed using ImageJ software (ImageJ, US National Institute of Health, Bethesda, MD, USA).

242

243 *Flow cytometric confirmation of EEC phenotype*

244 EECs were blocked with 10% normal goat serum for 10 minutes on ice. Cells were then either left
245 unstained (negative control) or incubated with mouse anti-human E-cadherin-BV421 (BD
246 Biosciences, Oxford, UK), mouse anti-human EpCAM-PE (Abcam, Cambridge, UK), mouse anti-
247 human CD31-PerCP-Cy5.5 and rat anti-human CD45-APC-Cy7 (BioLegend, London, UK). Flow
248 cytometry was carried out on a BD LSR Fortessa 5L flow cytometer (BD Biosciences, Oxford, UK).
249 Analysis was carried using FlowJo software (BD Biosciences, Oxford, UK).

250

251 *Generation of a 3D in-vitro organotypic model of a receptive endometrium*

252 EECs were primed in ESC medium (containing 1 μg/ml insulin and 0.3 ng/ml E2) for 48 hours at 37°C
253 in an atmosphere of 5% CO_2. When the EECs had already been incubating with ESC medium 24
254 hours, St-T1b cells were seeded at a ratio of 1:3 growth factor-reduced (GFR) Matrigel (BD
255 Biosciences, Oxford, UK) in ESC medium, at a density of 6 x 10^6 cells/ml, 60 μl/well (3.6 x 10^5
256 cells/well) in wells of a 96-well plate, and allowed to set into a 3D structure at 37°C in an atmosphere
257 of 5% CO_2 over 45 minutes. A further 200 μl ESC medium was subsequently added to wells and
258 maintained overnight at 37°C in an atmosphere of 5% CO_2. Once the EECs had been primed in ESC
259 medium for 48 hours, and the St-T1b cells grown within GFR Matrigel overnight, the medium was
260 removed from wells containing the St-T1b 3D structures, and EECs were trypsinated, washed,
261 resuspended in ESC medium and seeded on top of the 3D St-T1b cells at a density of 1 x 10^7 cells/ml,
262 100 μl/well (1 x 10^6 cells/well). To confirm that the phenotypic changes resulting from the hormonal
263 stimulation were dependent on cell-to-cell communication between the stromal and epithelial
264 compartments in our model, a parallel group was included, in which EECs were cultured alone
265 without ESCs on GFR Matrigel-coated plates. Cells were further incubated overnight at 37°C in an
266 atmosphere of 5% CO_2. Following overnight incubation, hormonal stimuli were added to cells every
267 48 hours as described in Table 1, with the first addition of stimuli considered as day 0. Cells were
268 fixed with 4% PFA on days 4, 6 and 8 for quantitative in-cell western analyses.

269

270 *Quantification of integrin αVβ3 expression by in-cell western assay*

271 Fixed cells were blocked overnight with Odyssey® buffer (LI-COR Biosciences, Cambridge, UK),
272 followed by incubation with mouse anti-human integrin αVβ3 (Abcam, Cambridge, UK; ab190147;
273 1:100), overnight at 4°C. Cells were then washed with PBS and the subsequent protocol, using a
274 goat anti-mouse IRDye® 800CW antibody and the CellTag™ 700 normalisation stain (LI-COR

Biosciences, Cambridge, UK), was carried out according to the manufacturer's instructions. Cells were imaged and analysed using the Odyssey CLx Near-Infrared Fluorescence Imaging System (LI-COR Biosciences, Cambridge, UK). In each experiment, data were normalised such that the integrin αVβ3 expression in the control (unstimulated) wells were given a value of 100.

Statistical analysis

One-way ANOVA with Tukey's multiple comparison post-test was used to determine *P* values using GraphPad Prism. $P < 0.05$ was considered statistically significant.

Results

Decidualisation induces the transient upregulation of ST2L in St-T1b cells

Decidualisation was induced in St-T1b cells with increasing levels of MPA, E2 and cAMP over 8 days (Table 1), to model the rising progesterone and estrogen levels that drive the structural and functional changes in the secretory stage endometrium. Transformation of the St-T1b cells into characteristically larger, rounded cells, 'decidualised' stromal cells, with increased cytoplasmic and nuclear size, was observed. Decidualisation was confirmed by immunocytochemical analyses of insulin-like growth factor-binding protein-1 (IGFBP-1) expression (Figure 1A). IGFBP-1 is a widely used marker to assess the differentiation status and quality of the decidual response of ESCs in culture (Gao, Mazella et al. 1994, Giudice, Mark et al. 1998, Fazleabas, Kim et al. 2004, Kim, Taylor et al. 2007, Samalecos, Reimann et al. 2009, Gellersen and Brosens 2014, Tamura, Jozaki et al. 2018). Furthermore, decidual transformation of ESCs was additionally corroborated by visible enlargement and rounding of the nucleus and an expanding cytoplasm upon hormonal stimulation, which also induced a transient upregulation of the interleukin-33 (IL-33) transmembrane receptor, ST2L, on day 6 (Figure 1B), indicating a transient autoinflammatory decidual response.

Primary human endometrial epithelial cells were rapidly expanded in vitro, by conditional reprogramming, with retention of epithelial markers cells

Epithelial cells can be rapidly expanded *in vitro*, by conditional reprogramming, with the use of a Rho kinase inhibitor (Y-27632) and irradiated fibroblast feeder cells (Liu, Ory et al. 2012, Suprynowicz, Upadhyay et al. 2012, Palechor-Ceron, Suprynowicz et al. 2013). This technique was adapted for human EECs in the current study, following isolation from clinical endometrial biopsies by tissue digestion with collagenase and DNase, and separation from ESCs (Figure 2A). Conditionally

reprogrammed EECs were generated, and these cells could be passaged several times with retention of epithelial markers. The conditionally reprogrammed EECs could be cryopreserved in single cell suspension in mFreSR™1 freezing medium and successfully revived following cryopreservation. EEC phenotype was confirmed by immunocytochemical expression of the epithelial marker cytokeratin-18 (CK-18) and absence of the stromal cell marker vimentin (Figure 2B). Further validation of an epithelial phenotype was conducted by flow cytometric analyses, which demonstrated that the cells expressed two additional epithelial markers, EpCAM and E-cadherin, but did not express the leukocyte and endothelial cell markers, CD45 and CD31, respectively (Figure 2C).

Generation of a novel endometrial organotypic in vitro co-culture model of the 'window of implantation'

Firstly, EECs and ESCs were primed in medium containing with E2 and insulin to model the proliferative stage of the uterine cycle. EECs were then co-cultured with St-T1b cells in a 3D structure (Figure 3A), to produce an endometrial organotypic co-culture model. The organotypic 3D co-cultures were subjected to decidualisation hormonal stimuli over 8 days (Table 1), to model the secretory stage of the uterine cycle and ultimately a receptive endometrial phenotype. EECs were monitored for expression of integrin αVβ3 (a key marker of uterine receptivity) by quantitative immunocytochemical detection, and in-cell western analyses demonstrated that integrin αVβ3 expression by EECs was significantly higher on day 8 after hormonal stimulation compared to basal expression where the cells did not receive any hormonal stimuli ($P < 0.0005$), as well as in comparison to integrin αVβ3 expression on day 4 of treatment ($P < 0.05$) (Figure 3B). There was no significant change over time in basal integrin αVβ3 expression in the control unstimulated group, and the data from the stimulated cells were therefore normalised to the control unstimulated group. Furthermore, there was no induction of epithelial αVβ3 expression in a parallel group in which EECs were cultured alone and subjected to hormonal stimuli over 8 days, indicating combined effects of hormone treatment and cell-to-cell communication between the stromal and epithelial compartments in our model (Supplementary figure 1).

Discussion

Decidualisation begins during the secretory phase of the menstrual cycle in response to rising steroid hormone levels, and is marked by the differentiation of fibroblast-like ESCs into specialised decidual cells, secretory changes in the uterine epithelial glands, the accumulation of uterine natural

killer cells, and vascular changes in the uterine spiral arteries (Maruyama and Yoshimura 2008, Cartwright, Fraser et al. 2010, Fraser, Whitley et al. 2015). These changes are not only important for implantation success, but defective endometrial receptivity is also associated with a wide range of gynaecological, reproductive, obstetric disorders, as well as in the pathophysiology reproductive malignancies (Makieva, Giacomini et al. 2018).

The key molecular players of ESC decidual transformation are progesterone and cAMP, which act synergistically to stimulate successful differentiation of ESCs into their decidualised state (Brar, Frank et al. 1997, Gellersen and Brosens 2003). Progesterone acts on ESCs by binding to the progesterone receptor (PR), a member of the steroid/thyroid hormone receptor superfamily of ligand-activated transcription factors (Gellersen and Brosens 2003). Estrogen is responsible for inducing PR expression in ESCs that determine progesterone responsiveness during the secretory stage of uterine cycle (Patel, Elguero et al. 2015). Rising progesterone levels drive the structural and biochemical changes from proliferative to secretory ESC status, with a simultaneous generation of endometrial receptivity and opening of the 'window of implantation' (Paulson 2011), and activation of the cAMP second messenger pathway can direct cellular specificity to progesterone action through the induction of diverse transcription factors that affect PR function (Gellersen and Brosens 2003). The initiation of endometrial receptivity is dependent on the local removal of steroid action in the endometrial epithelium, facilitated via selective downregulation of epithelial PRs and estrogen receptors (ERs), combined with the steroid-mediated paracrine effects from the stromal compartment. Through the selective epithelial cell PR and ER downregulation, it is believed that progesterone and estrogen act on stromal cells, which then influence epithelial cells through specific paracrine factors (Lessey, Ilesanmi et al. 1996, Lessey 1998). However, although adequate progesterone signalling is required to establish a receptive endometrial status, some studies suggest that untimely, excess progesterone can compromise decidualisation and endometrial receptivity (Labarta, Martínez-Conejero et al. 2011, Liang, Liu et al. 2018). Furthermore, while progesterone is responsible for the structural ESC changes during decidualisation, animal studies have demonstrated that uterine estrogen biosynthesis is crucial for the progression of decidualisation, possibly by promoting stromal cell gap junction communication, which is known to be implicated in preparing the endometrium for implantation (Ma, Song et al. 2003, Das, Mantena et al. 2009). Moreover, studies in mice have shown that estrogen is critical in regulating the receptive endometrial state; low estrogen levels can extend the 'window of implantation', whereas excessively high estrogen levels can promptly initiate a refractory state, indicating that a very narrow range of estrogen levels can determine the duration of endometrial receptivity, which could

have implications in the human setting (Ma, Song et al. 2003). Ovarian hormonal signalling must therefore be stringently regulated to establish an adequately programmed, appropriately timed receptive uterine environment to ensure pregnancy success, and to maintain gynaecological and reproductive health.

In the present study, decidualisation was induced with increasing doses of MPA, 8-Br-cAMP and E2 over 8 days, in order to recapitulate the time it takes for these functional changes to occur *in vivo*, since the 'window of implantation' becomes apparent (through detection of epithelial integrin αVβ3 expression) 6–8 days after ovulation (Lessey 1998). Frequently used *in vitro* decidualisation protocols include treatment of ESCs with constant doses of various combinations of progesterone or a progestin, a cAMP-inducing analogue and E2, with high variability in duration of treatment (Logan, Ponnampalam et al. 2013, Gellersen and Brosens 2014, Michalski, Chadchan et al. 2018). Early *in vitro* decidualisation studies established that progestins (such as MPA) induce enhanced decidual effects in cultured ESCs compared to progesterone, that a combination of progesterone with E2 can amplify decidual effects in ESCs compared to treatment with progesterone alone, and that the cAMP signal transduction cascade is a key stimulant in progesterone-dependent decidualisation (Eckert and Katzenellenbogen 1981, Irwin, Kirk et al. 1989, Levin, Tonetta et al. 1990, Gellersen, Kempf et al. 1994, Brar, Frank et al. 1997). Observations were based on physiological doses of ovarian hormones and cAMP stimulation that induced ESC ultrastructural and molecular changes characteristic of *in vivo* decidualisation (Eckert and Katzenellenbogen 1981, Irwin, Kirk et al. 1989, Gellersen, Kempf et al. 1994). While the majority of *in vitro* decidualisation protocols make use of continuous hormonal stimulatory doses (Logan, Ponnampalam et al. 2013, Gellersen and Brosens 2014, Michalski, Chadchan et al. 2018), we used increasing doses of hormonal and cAMP stimulation, to better represent the *in vivo* post-ovulatory rise in ovarian hormones and local cAMP production that controls decidualisation and endometrial receptivity. Our study demonstrates that these rising levels of ovarian hormones and cAMP can induce the transient upregulation of the IL-33 receptor, ST2L, which was not observed when ESCs were subjected to the standard continuous doses of hormonal and cAMP stimulation reported in the literature (Logan, Ponnampalam et al. 2013, Gellersen and Brosens 2014, Michalski, Chadchan et al. 2018) (Supplementary Figure 1). Decidualising ESCs have been shown to induce a transitory, acute autoinflammatory response, through secretion of IL-33 (a key regulator of the innate immune response), whilst upregulating the expression of its receptor, ST2L. This IL-33-ST2L signalling induces receptivity gene expression in the overlying epithelium, rendering the endometrium transiently receptive for the implantation of a

conceptus (Salker, Nautiyal et al. 2012). In the absence of an implanting conceptus, the ESCs mount an anti-inflammatory response that involves the downregulation of ST2L (Salker, Nautiyal et al. 2012).

Human studies are restricted by a lack of access to tissue throughout the different stages of the menstrual cycle. The current study provides a simple *in vitro* organotypic co-culture model of a 3D uterine structure, using Matrigel, an ESC cell line, and conditionally reprogrammed EECs. Matrigel is rich in laminin and collagen, bearing similarities to the uterine extracellular matrix composition (Tarrade, Goffin et al. 2002). Furthermore, it has been suggested that in addition to inducing a stromal regulatory phenotype, Matrigel is able to act as a mediator for ESCs to signal to EECs, in a similar paracrine manner to what occurs in living uterine tissue, when ESCs and EECs are co-cultured with Matrigel serving as the basement membrane (Arnold, Kaufman et al. 2001). The St-T1b ESC cell line used in our study expresses phenotypic ESC markers and can mimic primary decidual stromal cell responses *in vitro* (Samalecos, Reimann et al. 2009), and its use eliminates patient variability, as well as the possibility of 'contaminating' EECs being present within the stromal ESC component of the model. However, an EEC cell line was not utilised, as all commonly-used EEC cell lines are derived from malignant endometrial adenocarcinoma tissues. Since cancer cells have undergone numerous genetic and epigenetic alterations, adenocarcinoma-derived cell lines are not representative of non-cancerous biological processes such as decidualisation and the induction of endometrial receptivity. Conditional reprogramming transcends the difficulty of growing primary EECs in long-term culture, but allows propagation of primary epithelial cells into a highly proliferative state, whilst cells maintain their original karyotype and remain in a non-neoplastic state (Liu, Ory et al. 2012, Suprynowicz, Upadhyay et al. 2012, Palechor-Ceron, Suprynowicz et al. 2013). Cells are denoted as 'conditionally reprogrammed' due to the conditional induction of cell proliferation, with increased telomerase expression, by a combination of Y-27632 (which suppresses differentiation and extends life span in calcium- and serum-containing medium) and diffusible factor(s) released by the irradiation-induced apoptotic 3T3 feeder cells (Suprynowicz, Upadhyay et al. 2012, Palechor-Ceron, Suprynowicz et al. 2013). It has been suggested that the unrestricted cell proliferation induced by conditional reprogramming is mediated through the induction of telomerase and cytoskeletal remodelling and/or interference with the p16/Rb pathway (Liu, Ory et al. 2012, Palechor-Ceron, Suprynowicz et al. 2013). The capacity for rapid establishment of karyotype-stable cell cultures from normal human epithelium facilitates *in vitro* cellular studies without the drawbacks of cell cultures generated, for example, from induced pluripotent stem cells, such as genetic instability,

tumourigenicity and altered antigenicity (Suprynowicz, Upadhyay et al. 2012). Large numbers of EECs were generated through conditional reprogramming in the present study, which could be passaged multiple times with the retention of epithelial markers, thus providing the advantages of a conventional cell line.

The luminal epithelium is perceived as the fundamental site for endometrial receptivity (Idelevich and Vilella 2020), and integrin αVβ3 is a cell-surface adhesion receptor that appears on the apex of endometrial luminal epithelial cell surfaces, coincident with the 'window of implantation', and has putative roles in embryo attachment during implantation (Rai, Hopkisson et al. 1996, Apparao, Murray et al. 2001, Lessey 2002, Lessey and Castelbaum 2002). Integrin αVβ3 is maximally expressed during the 'window of implantation' (Apparao, Murray et al. 2001), and its endometrial expression is significantly lower in cases of unexplained infertility, indicating that aberrant epithelial integrin αVβ3 expression may be associated defective endometrial receptivity (Elnaggar, Farag et al. 2017). Here, we capitalise on the acute inflammatory initiation phase of decidual transformation that promotes the generation of endometrial receptivity. This temporal endometrial phenotypic change is not only important for implantation success, but its dysregulation is also associated with a wide range of gynaecological, reproductive, and obstetric disorders, as well as in the pathophysiology of reproductive malignancies (Makieva, Giacomini et al. 2018). It has been suggested that endometrial receptivity is mediated through both direct and indirect progesterone action (Lessey 2003). Epithelial steroid receptor expression varies during the menstrual cycle, with high PR levels in the proliferative phase and selective loss of epithelial PR (and reduced ER) expression in the secretory phase (Lessey, Ilesanmi et al. 1996), demonstrating a direct action of progesterone on epithelial cells. Endometrial receptivity is tightly associated with the shifts in PR and ER expression, which occur at the time of its onset around 5–6 days post-ovulation, concomitant with the appearance of epithelial integrin αVβ3 (Lessey 1998). Stromal cells, on the other hand, maintain their PR expression throughout the menstrual cycle, and progesterone action on stromal cells generates paracrine mediators (such as the secretion of specific growth factors, cytokines and inflammatory mediators) (Al-Sabbagh, Fusi et al. 2011, Salker, Nautiyal et al. 2012, Chen, Erikson et al. 2013) that promote epithelial gene expression, indicating the indirect action of progesterone, via stromal cells, in the induction of an epithelial receptive phenotype (Lessey 1998, Lessey 2003, Salker, Nautiyal et al. 2012). The addition of hormonal stimuli to our ESC-EEC co-culture system induced an autoinflammatory decidual stromal response and the upregulation of epithelial integrin αVβ3, representing phenotypic endometrial changes coincident with the 'window of implantation'.

Epithelial integrin αVβ3 expression within our 3D co-culture model coincided with the timing of the transient ST2L upregulation that was observed in ESCs, and was subsequently further amplified. Furthermore, there was no induction of epithelial integrin αVβ3 expression when EECs were cultured alone, without ESCs, and subjected to hormonal stimulation (Supplementary Figure 1), suggesting that the induction of epithelial integrin αVβ3 expression upon hormonal stimulation may have resulted from EEC-ESC crosstalk following IL-33-ST2L signalling within the stromal compartment, particularly since rising progesterone levels induce the downregulation of epithelial PR and ER expression during the secretory stage, permitting progesterone and estrogen to act on ESCs (Lessey, Ilesanmi et al. 1996, Lessey 1998). However, further experiments would be required to confirm whether these well-known endometrial functional changes are responsible for the lack of induction of integrin αVβ3 in EECs in monoculture, following hormonal stimulation, that was observed in the current study. In addition, differences were observed with modifications of decidualisation stimulation doses: while ESCs treated with increasing doses exceeding physiological hormonal and cAMP levels still elicited a transient ST2L upregulation, continuous stimulatory doses did not. Nonetheless, both of these stimulation protocols induced epithelial αVβ3 expression, albeit to a lesser amplitude (Supplementary Figure 1) than detected upon treatment with increasing physiological stimulatory doses. Such observations and nuances highlight the significance of appropriate experimental design, and also denote the importance of the interdependent relationship between the timing and level of ovarian hormonal signalling that is a likely requisite in the process of endometrial receptivity.

Limitations of the study

We acknowledge that the simplified functional endometrial organotypic model system presented here does not fully represent all the cellular components and communications that are implicated in the early events leading up to and during the 'window of implantation'. These include glandular epithelial cells that undergo secretory transformation to provide histiotrophic nutrition for implanting embryo, decidual natural killer cells that have important functions in stromal-immune crosstalk, uterine vascular development, embryo implantation and trophoblast invasion, or vascular components that undergo changes (Maruyama and Yoshimura 2008, Cartwright, Fraser et al. 2010, Weimar, Post Uiterweer et al. 2013, Fraser, Whitley et al. 2015). However, ESCs are the main cell-type in the uterine microenvironment, and through an initial acute autoinflammatory decidual response, they are pivotal for transforming the uterus into a receptive phenotype by signalling to

the overlying epithelium to induce the expression of key receptivity molecules. We have therefore put emphasis on the stromal and luminal epithelial components for the development of our organotypic model system, paracrine interactions of which are central to the generation of endometrial receptivity (Lessey 1998, Lessey 2003, Al-Sabbagh, Fusi et al. 2011, Salker, Nautiyal et al. 2012, Lucas, Dyer et al. 2016). In addition, our model could benefit from further validation, for example, via photomicrographic verification of accurately representative 3D spatial relationships of the cell-types, through characterisation of EEC and ESC steroid receptor expression upon hormonal stimulation, and by using the iGenomix® (iGenomix UK Ltd, Surrey, UK) Endometrial Receptivity Array (ERA) genomic tool (Katzorke, Vilella et al. 2016), for additional confirmation of a receptive endometrial phenotype.

Concluding remarks

Endometrial cell and molecular signalling errors are widely associated with uterine pathologies ranging from infertility to cancer (Makieva, Giacomini et al. 2018). Any disturbance in decidual transformation of the endometrium, and in turn endometrial receptivity, can cause endometrial functional inadequacy, leading to implantation failure or pregnancy loss resulting from abnormal implantation. Dysregulation of decidualisation and endometrial receptivity have been implicated in infertility, implantation failure, recurrent miscarriage, pre-eclampsia and intrauterine growth restriction (Norwitz 2006, Strowitzki, Germeyer et al. 2006, Cartwright, Fraser et al. 2010, Lessey 2011, Patel and Lessey 2011, Gellersen and Brosens 2014, Timeva, Shterev et al. 2014, Rabaglino, Post Uiterweer et al. 2015, Tan, Hang et al. 2015, Conrad, Rabaglino et al. 2017). In addition, several gynaecological disorders, including endometriosis, polycystic ovarian syndrome, hydrosalpinges and luteal phase defect, are also associated with decreased endometrial receptivity and anomalous expression of endometrial biomarkers (Donaghay and Lessey 2007). The endometrial organotypic system presented here may therefore facilitate a better understanding of interactions within the uterine microenvironment. These could include, for example, the immunomodulatory and vascular changes that are of critical importance during the secretory stage, as well as the application to the current model of previously described organoid systems, or embryo implantation and trophoblast invasion study protocols (Teklenburg, Salker et al. 2010, Fraser, Whitley et al. 2012, Wang, Pilla et al. 2012, Wallace, Host et al. 2013, James, Tun et al. 2016, Turco, Gardner et al. 2017), taking into consideration both the respective distinct stages of decidualisation and implantation in any future studies conducted. Other further potential applications would be for the development of advances in contraceptives, as well as to investigate how various drugs (such as those used in infertility or

chemotherapy treatments) may interfere with endometrial signalling pathways, particularly where human *in vivo* studies are not feasible. The co-culture system developed here, therefore has the scope to be applied in an extensive range of settings, allowing investigations for the comprehensive understanding of the molecular interactions and cellular consequences within the uterine microenvironment during this early transitory period, in the broad context of several of reproductive, obstetric and gynaecological pathologies.

Authors' roles

RF conceived the study. RF and RS performed experiments. RF analysed data, prepared the manuscript, and was responsible for funding acquisition to provide consumables. C-JL was responsible for funding acquisition to provide salary, space and equipment for this work to be conducted, and provided critical appraisal of the research. All authors revised the manuscript and approved the final version.

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Conflict of interest

None declared.

Data availability statement

The data underlying this article are available in the article and in its online supplementary material.

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Table and figure legends

Table 1: To produce a physiologically representative decidual response *in vitro*, cells were stimulated with minimal medium 1 (MM1) containing increasing concentrations of medroxyprogesterone acetate (MPA), 8-bromoadenosine 3',5'-cyclic adenosine monophosphate (8-Br-cAMP) and 17β-estradiol (E2), or MM1 with 0.001% ethanol (EtOH), every 48 hours, and monitored over 8 days.

Figure 1: Hormonal stimulation of St-T1b cells with medroxyprogesterone acetate, 8-bromoadenosine 3',5'-cyclic adenosine monophosphate and 17β-estradiol. Decidualisation confirmed by immunohistochemical detection of **[A]** IGFBP-1 expression, and **[B]** the transient upregulation of ST2L expression. Scale bars = 100 μm.

Figure 2: **[A]** Endometrial epithelial cells (EECs) were isolated from endometrial biopsies and expanded *in vitro* by conditional reprogramming. Confirmation of epithelial phenotype by **[B]** immunocytochemical analyses demonstrated cytokeratin-18 (CK18) expression and negative vimentin staining in EECs, with endometrial stromal cells (ESCs) serving as negative and positive controls, respectively. Scale bars = 100 μm. **[C]** Flow cytometric analyses demonstrated EpCAM and E-cadherin expression by EECs, but no CD31 and CD45 expression.

Figure 3. **[A]** To construct a 3D endometrial organotypic co-culture model, St-T1b cells were embedded in growth factor-reduced Matrigel, with a single layer of EECs seeded on top. **[B]** In-cell western® analysis was conducted to quantify epithelial integrin αVβ3 expression with or without treatment with medroxyprogesterone acetate, cyclic adenosine monophosphate and 17β-estradiol, on days 4, 6 and 8. Results are mean ± SEM of eight separate experiments. $^{**}P < 0.05$; $^{***}P < 0.0001$; one-way ANOVA with Tukey's multiple comparison post-test analysis.

A 3-dimensional endometrial organotypic model simulating the acute inflammatory decidualisation initiation phase with epithelial induction of the key endometrial receptivity marker, integrin αVβ3

Running title: 3D *in vitro* receptive endometrium

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Abstract

Study question: There is a distinct lack of directly relevant *in vitro* models of endometrial receptivity; is it possible to develop a simplified physiological *in vitro* system representing the key cell-types associated with a receptive endometrial phenotype?

Summary answer: We present a new concept to investigate endometrial receptivity, with a 3-dimensional (3D) organotypic co-culture model to simulate an early and transient acute autoinflammatory decidual status that resolves in the induction of a receptive endometrial phenotype.

What is known already: Embryo implantation is dependent on a receptive uterine environment. Ovarian steroids drive post-ovulation structural and functional changes in the endometrium, which becomes transiently receptive for an implanting conceptus, termed the 'window of implantation', and dysregulation of endometrial receptivity is implicated in a range of reproductive, obstetric, and gynaecological disorders and malignancies. The interactions that take place within the uterine microenvironment during this time are not fully understood, and human studies are constrained by a lack of access to uterine tissue from specific time-points during the menstrual cycle. Physiologically relevant *in vitro* model systems are therefore fundamental for conducting investigations to better understand the cellular and molecular mechanisms controlling endometrial receptivity.

Participants/materials, setting, methods: An endometrial stromal cell (ESC) line, and endometrial epithelial cells (EECs) isolated from uterine biopsy tissue and expanded *in vitro* by conditional reprogramming, were used throughout the study. Immunocytochemical and flow cytometric analyses were used to confirm epithelial phenotype following conditional reprogramming of EECs. To construct an endometrial organotypic co-culture model, ESCs were embedded within a 3D growth factor-reduced Matrigel structure, with a single layer of conditionally reprogrammed EECs seeded on top. Cells were stimulated with increasing doses of medroxyprogesterone acetate, cyclic adenosine monophosphate and estradiol, in order to induce ESC decidual transformation and endometrial receptivity. Decidual response and the induction of a receptive epithelial phenotype were assessed by immunocytochemical detection and quantitative in-cell western® analyses, respectively.

Main results and the role of chance: A transient upregulation of the IL-33 receptor, ST2L, was observed in ESCs, indicating a transient autoinflammatory decidual response to the hormonal stimulation, known to induce receptivity gene expression in the overlying epithelium. Hormonal stimulation induced the EEC expression of the key marker of endometrial receptivity, integrin αVβ3

(n = 8; *P <0.05; ***P < 0.0001). To our knowledge, this is the first demonstration of a dedicated endometrial organotypic model, that has been developed to investigate endometrial receptivity, via the recapitulation of an early decidual transitory acute autoinflammatory phase and induction of an epithelial phenotypic change, to represent a receptive endometrial status.

Limitations, reasons for caution: This simplified *in vitro* ESC-EEC co-culture system may be only partly representative of more complex *in vivo* conditions.

Wider implications of the findings: The 3D endometrial organotypic model presented here may offer a valuable tool for investigating a range of reproductive, obstetric, and gynaecological disorders, to improve outcomes for assisted reproductive technologies, and for the development of advances in contraceptive methods.

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Keywords: endometrial receptivity; window of implantation; decidualisation; endometrial organotypic model; conditional reprogramming

What does this mean for patients?

During the first stage of the menstrual cycle, estrogen secretion from the developing ovarian follicle promotes growth of the endometrium (the lining of the womb). Following ovulation, rising progesterone and estrogen levels, produced by the corpus luteum (the remaining structure of the ovarian follicle that contained the maturing egg before its release during ovulation), promote structural and functional changes in the endometrium, in preparation for the 'window of implantation' – a period of 2–5 days when the endometrium is optimally receptive to an implanting embryo. This period of optimal endometrial receptivity is not only crucial for successful embryo implantation, but abnormal molecular and cellular events in the endometrium during this transient period have been implicated in fertility problems, obstetric complications, gynaecological disorders, and endometrial cancer. In this study, we have developed a simplified cellular model, with physiologically appropriate hormonal stimulation, to investigate endometrial receptivity. A more comprehensive understanding of these events can lead to the development of new interventions to promote pregnancy success, long-term maternal and fetal health, women's health, as well as for improving contraceptive methods, and this new concept may be able to aid investigations to better understand the complex mechanisms involved in the generation of endometrial receptivity.

Introduction

Embryo implantation is a critical event in human pregnancy that is reliant on a receptive uterine environment. The cycling endometrium undergoes profound changes in women, leading to a carefully timed and defined period during which an embryo is able to attach and invade into a receptive uterus, resulting in the establishment of a successful pregnancy (Norwitz, Schust et al. 2001). Ovarian steroids, estrogen and progesterone, drive structural and functional changes in the uterine lining, preparing it for the implantation of a conceptus. The uterine lining, known as the endometrium, consists of a fibroblast-like stromal matrix lined by a single layer of columnar epithelium. Following ovulation, dynamic changes take place in the endometrial stromal cell (ESC) morphology, which undergo mesenchymal-to-epithelial transformation, and begin to differentiate into large, secretory, 'decidualised' stromal cells, in response to rising progesterone levels produced by the corpus luteum (Gellersen, Brosens et al. 2007, Salamonsen, Nie et al. 2009). Decidual transformation of ESCs is associated with enlargement and rounding of the nucleus, increased number of nucleoli, rough endoplasmic reticulum and Golgi complex expansion, and accumulation of glycogen and lipid droplets in the expanding cytoplasm (Gellersen and Brosens 2014, Kajihara, Tanaka et al. 2014, Okada, Tsuzuki et al. 2018).

Decidualisation is a dynamic, multistep progression of events, comprising 3 critical transitory phases: (i) an acute inflammatory initiation phase that subsequently transitions to (ii) an anti-inflammatory secretory phase during which time embryo implantation takes place, followed by (iii) a final resolution phase (Gellersen and Brosens 2014). First, ESCs undergo cell cycle exit at G_0/G_1 and mount a transient pro-inflammatory response generated by a self-limiting autoinflammatory response, which, in turn, results in the expression of key receptivity genes in the overlying endometrial surface luminal epithelium (Salker, Nautiyal et al. 2012). This renders the endometrium receptive for embryo implantation for a limited period of time: the 'window of implantation'. This period of optimal endometrial receptivity begins approximately 6 days post-ovulation and lasts 2–5 days (i.e. approximately between days 20 and 25 of an idealised 28-day cycle) (Denker 1993). A receptive endometrial phenotype is not only imperative for embryo implantation and pregnancy success, but aberrant decidual transformation and dysregulation of uterine receptivity have also been implicated in several obstetric complications, gynaecological disorders and cancer (Norwitz 2006, Strowitzki, Germeyer et al. 2006, Cartwright, Fraser et al. 2010, Lessey 2011, Patel and Lessey 2011, Gellersen and Brosens 2014, Timeva, Shterev et al. 2014, Rabaglino, Post Uiterweer et al. 2015, Tan, Hang et al. 2015, Conrad, Rabaglino et al. 2017).

Interactions between different cell-types have reciprocal effects on cell phenotypes and ensuing functions (Freshney 2005). The same is true for uterine compartments and the contributions of these interactions to endometrial receptivity, since decidual transformation of the stroma confers its ability to create paracrine gradients necessary for expression of evolutionarily conserved molecules by the luminal epithelium that are fundamental for embryo implantation (Achache and Revel 2006, Salker, Nautiyal et al. 2012). It has been demonstrated that endometrial receptivity is mediated by the activation of autoregulatory feedback loops in decidualising ESCs underlying the luminal epithelium, which activate the sequential expression of pro- and anti-inflammatory gene networks, and that ESCs can exert this function independent of local immune cells (Salker, Nautiyal et al. 2012). As such, it is evident that there is a co-dependent relationship between the endometrial stroma and epithelium, to prepare the uterus for pregnancy (Cakmak and Taylor 2011), with ESC decidual transformation being a prerequisite for the generation of endometrial receptivity (Vinketova, Mourdjeva et al. 2016, Yu, Berga et al. 2016). The current study focuses on the uterine phenotype during the acute inflammatory initiation phase of decidual transformation implicated in the generation of endometrial receptivity.

The mechanisms that control decidualisation and endometrial receptivity are highly complex, and we do not yet fully understand all the interactions that take place within the uterine microenvironment during this time. Uterine competence for embryo implantation sets the foundation for a successful pregnancy pathway; it is only when a clearer picture of the relative contributions of the cellular and molecular mechanisms leading to a receptive endometrial status become apparent, that the pathophysiology of several reproductive, obstetric and gynaecological disorders can be further defined, and appropriate interventions can be developed to promote pregnancy success as well as long-term maternal and fetal health. Likewise, a better understanding of these mechanisms will also be beneficial for innovations in contraceptive methods. We have developed a simplified 3-dimensional (3D) endometrial organotypic model to investigate endometrial receptivity, in which we simulate an early acute inflammatory endometrial status, that resolves in the generation of a receptive luminal epithelial phenotype, known as the 'window of implantation'. Organotypic culture refers to *in vitro* cell culture models in which two or more previously disaggregated cell-types are recombined in experimentally determined ratios and spatial relationships to reconstruct a constituent of the corresponding *in vivo* organ, as opposed to histiotypic cultures (high density culture of a single cell-type within a 3D matrix) or organoid cultures

(simplified, self-organising stem cell-derived 3D multicellular aggregates with the ability to mimic its *in vivo* organ counterpart) (Freshney 2005, Simian and Bissell 2017). An endometrial organotypic culture model, albeit a simplified representation, is able to better recapitulate the morphological and functional features of the *in vivo* uterine microenvironment, than is possible conventional 2-dimensional or even histiotypic cell culture methods. Physiologically representative *in vitro* model systems are vital for investigating the mechanisms implicated in endometrial receptivity, due to the ethical and logistical limitations of human studies. While there are reports of several *in vitro* endometrial co-culture models in the literature (Bentin-Ley, Horn et al. 2000, Arnold, Kaufman et al. 2001, Bläuer, Heinonen et al. 2005, Wang, Pilla et al. 2012, Chen, Erikson et al. 2013), these are not representative of the early acute inflammatory decidualisation phase that gives rise to a transiently receptive epithelial phenotype. The new functional co-culture system presented here may offer a convenient and accessible tool to improve our comprehension of interactions in the uterine microenvironment during this transitory phase.

Materials and methods

Culture and hormonal stimulation of the endometrial stromal cell line St-T1b a

The human ESC-derived telomerase-immortalised cell line, St-T1b (Samalecos, Reimann et al. 2009), kindly provided by Professor Jan Brosens (University of Warwick, UK), was maintained in phenol red-free Dulbecco's modified Eagle medium DMEM/Ham's F12 (DMEM/F12; Invitrogen, Renfrew, UK) with 10% steroid-depleted fetal calf serum (FCS) supplemented with 2mM L-glutamine, 1 µg/ml insulin, 0.3 ng/ml 17β-estradiol (E2), 50 µg/ml penicillin, 50 µg/ml streptomycin, and 0.2% Primocin (Invivogen, Toulouse, France) (ESC medium) at 37°C in an atmosphere of 5% CO_2. Phenol red-free medium was used in all experiments, due to phenol red's known estrogenic activity (Berthois, Katzenellenbogen et al. 1986). To induce decidualisation, cells were treated with minimal medium 1 (MM1; ESC medium without insulin and E2) containing increasing concentrations of the progestin, medroxyprogesterone acetate (MPA), 8-bromoadenosine 3',5'-cyclic adenosine monophosphate (8-Br-cAMP; Cambridge Bioscience, Cambridge, UK) and E2, or MM1 with 0.001% ethanol (EtOH) (Table 1) every 48 hours, and cultured over 8 days at 37°C in an atmosphere of 5% CO_2. All reagents for St-T1b cell culture were purchased from Sigma-Aldrich (Sigma-Aldrich, Dorset UK) unless stated otherwise. Cultured cells were fixed in 4% paraformaldehyde (PFA) on days 4, 6 and 8 for immunocytochemical analysis.

Isolation of epithelial cells from endometrial biopsies and ethical approval

Primary human endometrial biopsy tissue was obtained from three women attending gynaecological outpatient departments in NHS Lothian. Written informed consent was obtained from participants and ethical approval granted from Lothian Research Ethics Committee (REC 16/ES/0007). The women reported regular menstrual cycles and did not have exogenous hormone exposure for 2 months prior to biopsy. Women receiving hormonal therapy, suffering from endometriosis or those with fibroids of >3 cm were excluded. Human endometrial epithelial cells (EECs) were isolated from endometrial biopsies by tissue digestion and separation from ESCs. Endometrial tissue was minced using scalpel blades, followed by digestion with 100 μg/ml collagenase II and 0.25 μg/ml DNAse I (Sigma-Aldrich, Dorset UK) for 1.5 h at 37°C. The tissue homogenate was then sequentially strained through 70-μm and 40-μm membrane filters to separate glandular epithelium from ESCs. The membrane filters were back-washed with PBS to retrieve endometrial gland clumps, further rinsed with PBS to flush out any digestion medium, and mixed well to disperse clumps. EECs were then suspended in PBS and centrifuged at 500 x *g* for 5 minutes at room temperature (RT), supernatant subsequently discarded, followed by resuspension in PBS and centrifugation at 500 x *g* for 5 minutes at RT.

Expansion of endometrial epithelial cells by conditional reprogramming

EECs were rapidly expanded *in vitro* by conditional reprogramming with the use of Y-27632 (a Rho kinase inhibitor) and fibroblast feeder cells. First, 3T3 Swiss Albino fibroblasts (cell line obtained from the European Collection of Authenticated Cell Culture, Public Health England, Salisbury, UK) were grown in MM1 to approximately 80% confluence in T175 flasks, trypsinated, washed, resuspended in MM1 and irradiated at 30 Gy. The irradiated cells were washed, cultured at 37°C in an atmosphere of 5% CO_2, and conditioned medium collected 72 hours post-irradiation. EEC medium was prepared with phenol red-free DMEM/Ham's F12 containing 10% steroid-depleted FCS, and supplemented with 2mM L-glutamine, 5 μg/ml insulin, 24 μg/ml adenine, 0.4 μg/ml hydrocortisone, 10 ng/ml epidermal growth (EGF), 8.4 ng/ml cholera toxin, 10 μmol/l Y-27632 (Cambridge Bioscience, Cambridge, UK, 10 μg/ml gentamycin and 0.25 μg/ml amphotericin. The irradiated 3T3 conditioned medium was added to EEC medium in a 1:3 ratio, 1 part of IR 3T3 conditioned media to 3 parts of EEC medium; CREEC medium (conditional reprogramming EEC medium), and the EECs maintained in CREEC medium at 37°C in an atmosphere of 5% CO_2. This method has previously been shown to directly alter cell growth without selecting for a small sub-population of stem-like cells, while retaining a normal non-tumourigenic karyotype, and

conditionally inducing an indefinite proliferative state in primary mammalian epithelial cells (Liu, Ory et al. 2012, Suprynowicz, Upadhyay et al. 2012, Palechor-Ceron, Suprynowicz et al. 2013). All reagents for EEC culture and conditional reprogramming were purchased from Sigma-Aldrich (Sigma-Aldrich, Dorset UK) unless stated otherwise. EECs can be cryopreserved using mFreSR™ cryopreservation medium (Stemcell Technologies, Cambridge, UK). Cultured EECs were fixed in 4% PFA for immunocytochemical analysis.

Immunocytochemical confirmation of ESC decidualisation and EEC phenotype

Fixed cells (St-T1b cells and EECs) were permeabilised with 0.1% Triton X-100 in PBS for 10 minutes, and washed three times with 0.1% Tween-20 in PBS (PBST). Cells were then blocked with 5% BSA in PBS for 1 hour at RT, incubated with primary antibody overnight at 4°C, washed three times with 0.1% PBST, subsequently incubated with secondary antibody and 1:10,000 DAPI for 30 minutes at RT in the dark, followed by a final wash with PBS. Primary antibodies used were rabbit anti-human IGFBP-1 (Abcam, Cambridge, UK; ab111203; 1:100), rat anti-mouse ST2L (IL-33R/ST2) (eBioscience, Cheshire, UK; 17-9335-82; 1:100), rabbit anti-human cytokeratin-18 conjugated to phycoerythrin (Abcam, Cambridge, UK; ab218288; 1:1000), rabbit anti-human vimentin (New England Biolabs, Hitchin, UK; 5741; 1:100), and mouse anti-human integrin αVβ3 (Abcam, Cambridge, UK; ab190147; 1:100). A goat anti-rabbit antibody conjugated to Alexa Fluor 546 (Invitrogen, Renfrew, UK; A-11071; 1:300), a donkey anti-rabbit antibody conjugated to Alexa Fluor 568 (Invitrogen, Renfrew, UK; A10042; 1:250) and a donkey anti-mouse antibody conjugated to Alexa Fluor 488 (Invitrogen, Renfrew, UK; A21202; 1:500) were used as secondary antibodies. The cytokeratin-18 staining did not require incubation with a secondary antibody. The ST2L staining did not require cell permeabalisation for detection, but did require a signal amplification step after primary antibody incubation, with a biotinylated goat anti-rat antibody (Vector Laboratories, Peterborough, UK; BA-4000; 1:100) for 30 minutes at RT in the dark, followed by three washes with 0.1% PBST. Cells were then incubated with streptavidin conjugated to Alexa Fluor 488 (Invitrogen, Renfrew, UK; s11223; 1:200) and 1 mg/ml Hoechst 33342 (Invitrogen, Renfrew, UK; H3570) for 30 minutes at RT in the dark, and then washed with PBS. Imaging was conducted on an Olympus IX71 microscope with a QImaging optiMOS camera and CoolLED PE4000 light source (Olympus, Tokyo, Japan) or a Zeiss Axio Observer 7 microscope (Carl Zeiss Ltd, Cambridge, UK) with a Hamamatsu ORCA-Flash LT camera (Hamamatsu Protonics, Hertfordshire, UK) and Zeiss Colibri 7 LED light source (Carl Zeiss Ltd, Cambridge, UK). Images were analysed using ImageJ software (ImageJ, US National Institute of Health, Bethesda, MD, USA).

242

243 *Flow cytometric confirmation of EEC phenotype*

244 EECs were blocked with 10% normal goat serum for 10 minutes on ice. Cells were then either left
245 unstained (negative control) or incubated with mouse anti-human E-cadherin-BV421 (BD
246 Biosciences, Oxford, UK), mouse anti-human EpCAM-PE (Abcam, Cambridge, UK), mouse anti-
247 human CD31-PerCP-Cy5.5 and rat anti-human CD45-APC-Cy7 (BioLegend, London, UK). Flow
248 cytometry was carried out on a BD LSR Fortessa 5L flow cytometer (BD Biosciences, Oxford, UK).
249 Analysis was carried using FlowJo software (BD Biosciences, Oxford, UK).

250

251 *Generation of a 3D in-vitro organotypic model of a receptive endometrium*

252 EECs were primed in ESC medium (containing 1 μg/ml insulin and 0.3 ng/ml E2) for 48 hours at 37°C
253 in an atmosphere of 5% CO_2. When the EECs had already been incubating with ESC medium 24
254 hours, St-T1b cells were seeded at a ratio of 1:3 growth factor-reduced (GFR) Matrigel (BD
255 Biosciences, Oxford, UK) in ESC medium, at a density of 6 x 10^6 cells/ml, 60 μl/well (3.6 x 10^5
256 cells/well) in wells of a 96-well plate, and allowed to set into a 3D structure at 37°C in an atmosphere
257 of 5% CO_2 over 45 minutes. A further 200 μl ESC medium was subsequently added to wells and
258 maintained overnight at 37°C in an atmosphere of 5% CO_2. Once the EECs had been primed in ESC
259 medium for 48 hours, and the St-T1b cells grown within GFR Matrigel overnight, the medium was
260 removed from wells containing the St-T1b 3D structures, and EECs were trypsinated, washed,
261 resuspended in ESC medium and seeded on top of the 3D St-T1b cells at a density of 1 x 10^7 cells/ml,
262 100 μl/well (1 x 10^6 cells/well). To confirm that the phenotypic changes resulting from the hormonal
263 stimulation were dependent on cell-to-cell communication between the stromal and epithelial
264 compartments in our model, a parallel group was included, in which EECs were cultured alone
265 without ESCs on GFR Matrigel-coated plates. Cells were further incubated overnight at 37°C in an
266 atmosphere of 5% CO_2. Following overnight incubation, hormonal stimuli were added to cells every
267 48 hours as described in Table 1, with the first addition of stimuli considered as day 0. Cells were
268 fixed with 4% PFA on days 4, 6 and 8 for quantitative in-cell western analyses.

269

270 *Quantification of integrin αVβ3 expression by in-cell western assay*

271 Fixed cells were blocked overnight with Odyssey® buffer (LI-COR Biosciences, Cambridge, UK),
272 followed by incubation with mouse anti-human integrin αVβ3 (Abcam, Cambridge, UK; ab190147;
273 1:100), overnight at 4°C. Cells were then washed with PBS and the subsequent protocol, using a
274 goat anti-mouse IRDye® 800CW antibody and the CellTag™ 700 normalisation stain (LI-COR

Biosciences, Cambridge, UK), was carried out according to the manufacturer's instructions. Cells were imaged and analysed using the Odyssey CLx Near-Infrared Fluorescence Imaging System (LI-COR Biosciences, Cambridge, UK). In each experiment, data were normalised such that the integrin αVβ3 expression in the control (unstimulated) wells were given a value of 100.

Statistical analysis

One-way ANOVA with Tukey's multiple comparison post-test was used to determine *P* values using GraphPad Prism. $P < 0.05$ was considered statistically significant.

Results

Decidualisation induces the transient upregulation of ST2L in St-T1b cells

Decidualisation was induced in St-T1b cells with increasing levels of MPA, E2 and cAMP over 8 days (Table 1), to model the rising progesterone and estrogen levels that drive the structural and functional changes in the secretory stage endometrium. Transformation of the St-T1b cells into characteristically larger, rounded cells, 'decidualised' stromal cells, with increased cytoplasmic and nuclear size, was observed. Decidualisation was confirmed by immunocytochemical analyses of insulin-like growth factor-binding protein-1 (IGFBP-1) expression (Figure 1A). IGFBP-1 is a widely used marker to assess the differentiation status and quality of the decidual response of ESCs in culture (Gao, Mazella et al. 1994, Giudice, Mark et al. 1998, Fazleabas, Kim et al. 2004, Kim, Taylor et al. 2007, Samalecos, Reimann et al. 2009, Gellersen and Brosens 2014, Tamura, Jozaki et al. 2018). Furthermore, decidual transformation of ESCs was additionally corroborated by visible enlargement and rounding of the nucleus and an expanding cytoplasm upon hormonal stimulation, which also induced a transient upregulation of the interleukin-33 (IL-33) transmembrane receptor, ST2L, on day 6 (Figure 1B), indicating a transient autoinflammatory decidual response.

Primary human endometrial epithelial cells were rapidly expanded in vitro, by conditional reprogramming, with retention of epithelial markers cells

Epithelial cells can be rapidly expanded *in vitro*, by conditional reprogramming, with the use of a Rho kinase inhibitor (Y-27632) and irradiated fibroblast feeder cells (Liu, Ory et al. 2012, Suprynowicz, Upadhyay et al. 2012, Palechor-Ceron, Suprynowicz et al. 2013). This technique was adapted for human EECs in the current study, following isolation from clinical endometrial biopsies by tissue digestion with collagenase and DNase, and separation from ESCs (Figure 2A). Conditionally

reprogrammed EECs were generated, and these cells could be passaged several times with retention of epithelial markers. The conditionally reprogrammed EECs could be cryopreserved in single cell suspension in mFreSR™1 freezing medium and successfully revived following cryopreservation. EEC phenotype was confirmed by immunocytochemical expression of the epithelial marker cytokeratin-18 (CK-18) and absence of the stromal cell marker vimentin (Figure 2B). Further validation of an epithelial phenotype was conducted by flow cytometric analyses, which demonstrated that the cells expressed two additional epithelial markers, EpCAM and E-cadherin, but did not express the leukocyte and endothelial cell markers, CD45 and CD31, respectively (Figure 2C).

Generation of a novel endometrial organotypic in vitro co-culture model of the 'window of implantation'

Firstly, EECs and ESCs were primed in medium containing with E2 and insulin to model the proliferative stage of the uterine cycle. EECs were then co-cultured with St-T1b cells in a 3D structure (Figure 3A), to produce an endometrial organotypic co-culture model. The organotypic 3D co-cultures were subjected to decidualisation hormonal stimuli over 8 days (Table 1), to model the secretory stage of the uterine cycle and ultimately a receptive endometrial phenotype. EECs were monitored for expression of integrin αVβ3 (a key marker of uterine receptivity) by quantitative immunocytochemical detection, and in-cell western analyses demonstrated that integrin αVβ3 expression by EECs was significantly higher on day 8 after hormonal stimulation compared to basal expression where the cells did not receive any hormonal stimuli ($P < 0.0005$), as well as in comparison to integrin αVβ3 expression on day 4 of treatment ($P < 0.05$) (Figure 3B). There was no significant change over time in basal integrin αVβ3 expression in the control unstimulated group, and the data from the stimulated cells were therefore normalised to the control unstimulated group. Furthermore, there was no induction of epithelial αVβ3 expression in a parallel group in which EECs were cultured alone and subjected to hormonal stimuli over 8 days, indicating combined effects of hormone treatment and cell-to-cell communication between the stromal and epithelial compartments in our model (Supplementary figure 1).

Discussion

Decidualisation begins during the secretory phase of the menstrual cycle in response to rising steroid hormone levels, and is marked by the differentiation of fibroblast-like ESCs into specialised decidual cells, secretory changes in the uterine epithelial glands, the accumulation of uterine natural

killer cells, and vascular changes in the uterine spiral arteries (Maruyama and Yoshimura 2008, Cartwright, Fraser et al. 2010, Fraser, Whitley et al. 2015). These changes are not only important for implantation success, but defective endometrial receptivity is also associated with a wide range of gynaecological, reproductive, obstetric disorders, as well as in the pathophysiology reproductive malignancies (Makieva, Giacomini et al. 2018).

The key molecular players of ESC decidual transformation are progesterone and cAMP, which act synergistically to stimulate successful differentiation of ESCs into their decidualised state (Brar, Frank et al. 1997, Gellersen and Brosens 2003). Progesterone acts on ESCs by binding to the progesterone receptor (PR), a member of the steroid/thyroid hormone receptor superfamily of ligand-activated transcription factors (Gellersen and Brosens 2003). Estrogen is responsible for inducing PR expression in ESCs that determine progesterone responsiveness during the secretory stage of uterine cycle (Patel, Elguero et al. 2015). Rising progesterone levels drive the structural and biochemical changes from proliferative to secretory ESC status, with a simultaneous generation of endometrial receptivity and opening of the 'window of implantation' (Paulson 2011), and activation of the cAMP second messenger pathway can direct cellular specificity to progesterone action through the induction of diverse transcription factors that affect PR function (Gellersen and Brosens 2003). The initiation of endometrial receptivity is dependent on the local removal of steroid action in the endometrial epithelium, facilitated via selective downregulation of epithelial PRs and estrogen receptors (ERs), combined with the steroid-mediated paracrine effects from the stromal compartment. Through the selective epithelial cell PR and ER downregulation, it is believed that progesterone and estrogen act on stromal cells, which then influence epithelial cells through specific paracrine factors (Lessey, Ilesanmi et al. 1996, Lessey 1998). However, although adequate progesterone signalling is required to establish a receptive endometrial status, some studies suggest that untimely, excess progesterone can compromise decidualisation and endometrial receptivity (Labarta, Martínez-Conejero et al. 2011, Liang, Liu et al. 2018). Furthermore, while progesterone is responsible for the structural ESC changes during decidualisation, animal studies have demonstrated that uterine estrogen biosynthesis is crucial for the progression of decidualisation, possibly by promoting stromal cell gap junction communication, which is known to be implicated in preparing the endometrium for implantation (Ma, Song et al. 2003, Das, Mantena et al. 2009). Moreover, studies in mice have shown that estrogen is critical in regulating the receptive endometrial state; low estrogen levels can extend the 'window of implantation', whereas excessively high estrogen levels can promptly initiate a refractory state, indicating that a very narrow range of estrogen levels can determine the duration of endometrial receptivity, which could

have implications in the human setting (Ma, Song et al. 2003). Ovarian hormonal signalling must therefore be stringently regulated to establish an adequately programmed, appropriately timed receptive uterine environment to ensure pregnancy success, and to maintain gynaecological and reproductive health.

In the present study, decidualisation was induced with increasing doses of MPA, 8-Br-cAMP and E2 over 8 days, in order to recapitulate the time it takes for these functional changes to occur *in vivo*, since the 'window of implantation' becomes apparent (through detection of epithelial integrin αVβ3 expression) 6–8 days after ovulation (Lessey 1998). Frequently used *in vitro* decidualisation protocols include treatment of ESCs with constant doses of various combinations of progesterone or a progestin, a cAMP-inducing analogue and E2, with high variability in duration of treatment (Logan, Ponnampalam et al. 2013, Gellersen and Brosens 2014, Michalski, Chadchan et al. 2018). Early *in vitro* decidualisation studies established that progestins (such as MPA) induce enhanced decidual effects in cultured ESCs compared to progesterone, that a combination of progesterone with E2 can amplify decidual effects in ESCs compared to treatment with progesterone alone, and that the cAMP signal transduction cascade is a key stimulant in progesterone-dependent decidualisation (Eckert and Katzenellenbogen 1981, Irwin, Kirk et al. 1989, Levin, Tonetta et al. 1990, Gellersen, Kempf et al. 1994, Brar, Frank et al. 1997). Observations were based on physiological doses of ovarian hormones and cAMP stimulation that induced ESC ultrastructural and molecular changes characteristic of *in vivo* decidualisation (Eckert and Katzenellenbogen 1981, Irwin, Kirk et al. 1989, Gellersen, Kempf et al. 1994). While the majority of *in vitro* decidualisation protocols make use of continuous hormonal stimulatory doses (Logan, Ponnampalam et al. 2013, Gellersen and Brosens 2014, Michalski, Chadchan et al. 2018), we used increasing doses of hormonal and cAMP stimulation, to better represent the *in vivo* post-ovulatory rise in ovarian hormones and local cAMP production that controls decidualisation and endometrial receptivity. Our study demonstrates that these rising levels of ovarian hormones and cAMP can induce the transient upregulation of the IL-33 receptor, ST2L, which was not observed when ESCs were subjected to the standard continuous doses of hormonal and cAMP stimulation reported in the literature (Logan, Ponnampalam et al. 2013, Gellersen and Brosens 2014, Michalski, Chadchan et al. 2018) (Supplementary Figure 1). Decidualising ESCs have been shown to induce a transitory, acute autoinflammatory response, through secretion of IL-33 (a key regulator of the innate immune response), whilst upregulating the expression of its receptor, ST2L. This IL-33-ST2L signalling induces receptivity gene expression in the overlying epithelium, rendering the endometrium transiently receptive for the implantation of a

conceptus (Salker, Nautiyal et al. 2012). In the absence of an implanting conceptus, the ESCs mount an anti-inflammatory response that involves the downregulation of ST2L (Salker, Nautiyal et al. 2012).

Human studies are restricted by a lack of access to tissue throughout the different stages of the menstrual cycle. The current study provides a simple *in vitro* organotypic co-culture model of a 3D uterine structure, using Matrigel, an ESC cell line, and conditionally reprogrammed EECs. Matrigel is rich in laminin and collagen, bearing similarities to the uterine extracellular matrix composition (Tarrade, Goffin et al. 2002). Furthermore, it has been suggested that in addition to inducing a stromal regulatory phenotype, Matrigel is able to act as a mediator for ESCs to signal to EECs, in a similar paracrine manner to what occurs in living uterine tissue, when ESCs and EECs are co-cultured with Matrigel serving as the basement membrane (Arnold, Kaufman et al. 2001). The St-T1b ESC cell line used in our study expresses phenotypic ESC markers and can mimic primary decidual stromal cell responses *in vitro* (Samalecos, Reimann et al. 2009), and its use eliminates patient variability, as well as the possibility of 'contaminating' EECs being present within the stromal ESC component of the model. However, an EEC cell line was not utilised, as all commonly-used EEC cell lines are derived from malignant endometrial adenocarcinoma tissues. Since cancer cells have undergone numerous genetic and epigenetic alterations, adenocarcinoma-derived cell lines are not representative of non-cancerous biological processes such as decidualisation and the induction of endometrial receptivity. Conditional reprogramming transcends the difficulty of growing primary EECs in long-term culture, but allows propagation of primary epithelial cells into a highly proliferative state, whilst cells maintain their original karyotype and remain in a non-neoplastic state (Liu, Ory et al. 2012, Suprynowicz, Upadhyay et al. 2012, Palechor-Ceron, Suprynowicz et al. 2013). Cells are denoted as 'conditionally reprogrammed' due to the conditional induction of cell proliferation, with increased telomerase expression, by a combination of Y-27632 (which suppresses differentiation and extends life span in calcium- and serum-containing medium) and diffusible factor(s) released by the irradiation-induced apoptotic 3T3 feeder cells (Suprynowicz, Upadhyay et al. 2012, Palechor-Ceron, Suprynowicz et al. 2013). It has been suggested that the unrestricted cell proliferation induced by conditional reprogramming is mediated through the induction of telomerase and cytoskeletal remodelling and/or interference with the p16/Rb pathway (Liu, Ory et al. 2012, Palechor-Ceron, Suprynowicz et al. 2013). The capacity for rapid establishment of karyotype-stable cell cultures from normal human epithelium facilitates *in vitro* cellular studies without the drawbacks of cell cultures generated, for example, from induced pluripotent stem cells, such as genetic instability,

tumourigenicity and altered antigenicity (Suprynowicz, Upadhyay et al. 2012). Large numbers of EECs were generated through conditional reprogramming in the present study, which could be passaged multiple times with the retention of epithelial markers, thus providing the advantages of a conventional cell line.

The luminal epithelium is perceived as the fundamental site for endometrial receptivity (Idelevich and Vilella 2020), and integrin αVβ3 is a cell-surface adhesion receptor that appears on the apex of endometrial luminal epithelial cell surfaces, coincident with the 'window of implantation', and has putative roles in embryo attachment during implantation (Rai, Hopkisson et al. 1996, Apparao, Murray et al. 2001, Lessey 2002, Lessey and Castelbaum 2002). Integrin αVβ3 is maximally expressed during the 'window of implantation' (Apparao, Murray et al. 2001), and its endometrial expression is significantly lower in cases of unexplained infertility, indicating that aberrant epithelial integrin αVβ3 expression may be associated defective endometrial receptivity (Elnaggar, Farag et al. 2017). Here, we capitalise on the acute inflammatory initiation phase of decidual transformation that promotes the generation of endometrial receptivity. This temporal endometrial phenotypic change is not only important for implantation success, but its dysregulation is also associated with a wide range of gynaecological, reproductive, and obstetric disorders, as well as in the pathophysiology of reproductive malignancies (Makieva, Giacomini et al. 2018). It has been suggested that endometrial receptivity is mediated through both direct and indirect progesterone action (Lessey 2003). Epithelial steroid receptor expression varies during the menstrual cycle, with high PR levels in the proliferative phase and selective loss of epithelial PR (and reduced ER) expression in the secretory phase (Lessey, Ilesanmi et al. 1996), demonstrating a direct action of progesterone on epithelial cells. Endometrial receptivity is tightly associated with the shifts in PR and ER expression, which occur at the time of its onset around 5–6 days post-ovulation, concomitant with the appearance of epithelial integrin αVβ3 (Lessey 1998). Stromal cells, on the other hand, maintain their PR expression throughout the menstrual cycle, and progesterone action on stromal cells generates paracrine mediators (such as the secretion of specific growth factors, cytokines and inflammatory mediators) (Al-Sabbagh, Fusi et al. 2011, Salker, Nautiyal et al. 2012, Chen, Erikson et al. 2013) that promote epithelial gene expression, indicating the indirect action of progesterone, via stromal cells, in the induction of an epithelial receptive phenotype (Lessey 1998, Lessey 2003, Salker, Nautiyal et al. 2012). The addition of hormonal stimuli to our ESC-EEC co-culture system induced an autoinflammatory decidual stromal response and the upregulation of epithelial integrin αVβ3, representing phenotypic endometrial changes coincident with the 'window of implantation'.

Epithelial integrin αVβ3 expression within our 3D co-culture model coincided with the timing of the transient ST2L upregulation that was observed in ESCs, and was subsequently further amplified. Furthermore, there was no induction of epithelial integrin αVβ3 expression when EECs were cultured alone, without ESCs, and subjected to hormonal stimulation (Supplementary Figure 1), suggesting that the induction of epithelial integrin αVβ3 expression upon hormonal stimulation may have resulted from EEC-ESC crosstalk following IL-33-ST2L signalling within the stromal compartment, particularly since rising progesterone levels induce the downregulation of epithelial PR and ER expression during the secretory stage, permitting progesterone and estrogen to act on ESCs (Lessey, Ilesanmi et al. 1996, Lessey 1998). However, further experiments would be required to confirm whether these well-known endometrial functional changes are responsible for the lack of induction of integrin αVβ3 in EECs in monoculture, following hormonal stimulation, that was observed in the current study. In addition, differences were observed with modifications of decidualisation stimulation doses: while ESCs treated with increasing doses exceeding physiological hormonal and cAMP levels still elicited a transient ST2L upregulation, continuous stimulatory doses did not. Nonetheless, both of these stimulation protocols induced epithelial αVβ3 expression, albeit to a lesser amplitude (Supplementary Figure 1) than detected upon treatment with increasing physiological stimulatory doses. Such observations and nuances highlight the significance of appropriate experimental design, and also denote the importance of the interdependent relationship between the timing and level of ovarian hormonal signalling that is a likely requisite in the process of endometrial receptivity.

Limitations of the study

We acknowledge that the simplified functional endometrial organotypic model system presented here does not fully represent all the cellular components and communications that are implicated in the early events leading up to and during the 'window of implantation'. These include glandular epithelial cells that undergo secretory transformation to provide histiotrophic nutrition for implanting embryo, decidual natural killer cells that have important functions in stromal-immune crosstalk, uterine vascular development, embryo implantation and trophoblast invasion, or vascular components that undergo changes (Maruyama and Yoshimura 2008, Cartwright, Fraser et al. 2010, Weimar, Post Uiterweer et al. 2013, Fraser, Whitley et al. 2015). However, ESCs are the main cell-type in the uterine microenvironment, and through an initial acute autoinflammatory decidual response, they are pivotal for transforming the uterus into a receptive phenotype by signalling to

the overlying epithelium to induce the expression of key receptivity molecules. We have therefore put emphasis on the stromal and luminal epithelial components for the development of our organotypic model system, paracrine interactions of which are central to the generation of endometrial receptivity (Lessey 1998, Lessey 2003, Al-Sabbagh, Fusi et al. 2011, Salker, Nautiyal et al. 2012, Lucas, Dyer et al. 2016). In addition, our model could benefit from further validation, for example, via photomicrographic verification of accurately representative 3D spatial relationships of the cell-types, through characterisation of EEC and ESC steroid receptor expression upon hormonal stimulation, and by using the iGenomix® (iGenomix UK Ltd, Surrey, UK) Endometrial Receptivity Array (ERA) genomic tool (Katzorke, Vilella et al. 2016), for additional confirmation of a receptive endometrial phenotype.

Concluding remarks

Endometrial cell and molecular signalling errors are widely associated with uterine pathologies ranging from infertility to cancer (Makieva, Giacomini et al. 2018). Any disturbance in decidual transformation of the endometrium, and in turn endometrial receptivity, can cause endometrial functional inadequacy, leading to implantation failure or pregnancy loss resulting from abnormal implantation. Dysregulation of decidualisation and endometrial receptivity have been implicated in infertility, implantation failure, recurrent miscarriage, pre-eclampsia and intrauterine growth restriction (Norwitz 2006, Strowitzki, Germeyer et al. 2006, Cartwright, Fraser et al. 2010, Lessey 2011, Patel and Lessey 2011, Gellersen and Brosens 2014, Timeva, Shterev et al. 2014, Rabaglino, Post Uiterweer et al. 2015, Tan, Hang et al. 2015, Conrad, Rabaglino et al. 2017). In addition, several gynaecological disorders, including endometriosis, polycystic ovarian syndrome, hydrosalpinges and luteal phase defect, are also associated with decreased endometrial receptivity and anomalous expression of endometrial biomarkers (Donaghay and Lessey 2007). The endometrial organotypic system presented here may therefore facilitate a better understanding of interactions within the uterine microenvironment. These could include, for example, the immunomodulatory and vascular changes that are of critical importance during the secretory stage, as well as the application to the current model of previously described organoid systems, or embryo implantation and trophoblast invasion study protocols (Teklenburg, Salker et al. 2010, Fraser, Whitley et al. 2012, Wang, Pilla et al. 2012, Wallace, Host et al. 2013, James, Tun et al. 2016, Turco, Gardner et al. 2017), taking into consideration both the respective distinct stages of decidualisation and implantation in any future studies conducted. Other further potential applications would be for the development of advances in contraceptives, as well as to investigate how various drugs (such as those used in infertility or

chemotherapy treatments) may interfere with endometrial signalling pathways, particularly where human *in vivo* studies are not feasible. The co-culture system developed here, therefore has the scope to be applied in an extensive range of settings, allowing investigations for the comprehensive understanding of the molecular interactions and cellular consequences within the uterine microenvironment during this early transitory period, in the broad context of several of reproductive, obstetric and gynaecological pathologies.

Authors' roles

RF conceived the study. RF and RS performed experiments. RF analysed data, prepared the manuscript, and was responsible for funding acquisition to provide consumables. C-JL was responsible for funding acquisition to provide salary, space and equipment for this work to be conducted, and provided critical appraisal of the research. All authors revised the manuscript and approved the final version.

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Conflict of interest

None declared.

Data availability statement

The data underlying this article are available in the article and in its online supplementary material.

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768

769 ## Table and figure legends

770 Table 1: To produce a physiologically representative decidual response *in vitro*, cells were stimulated
771 with minimal medium 1 (MM1) containing increasing concentrations of medroxyprogesterone
772 acetate (MPA), 8-bromoadenosine 3',5'-cyclic adenosine monophosphate (8-Br-cAMP) and 17β-
773 estradiol (E2), or MM1 with 0.001% ethanol (EtOH), every 48 hours, and monitored over 8 days.

774

775 Figure 1: Hormonal stimulation of St-T1b cells with medroxyprogesterone acetate, 8-
776 bromoadenosine 3',5'-cyclic adenosine monophosphate and 17β-estradiol. Decidualisation
777 confirmed by immunohistochemical detection of **[A]** IGFBP-1 expression, and **[B]** the transient
778 upregulation of ST2L expression. Scale bars = 100 μm.

779

780 Figure 2: **[A]** Endometrial epithelial cells (EECs) were isolated from endometrial biopsies and
781 expanded *in vitro* by conditional reprogramming. Confirmation of epithelial phenotype by **[B]**
782 immunocytochemical analyses demonstrated cytokeratin-18 (CK18) expression and negative
783 vimentin staining in EECs, with endometrial stromal cells (ESCs) serving as negative and positive
784 controls, respectively. Scale bars = 100 μm. **[C]** Flow cytometric analyses demonstrated EpCAM and
785 E-cadherin expression by EECs, but no CD31 and CD45 expression.

786

787 Figure 3. **[A]** To construct a 3D endometrial organotypic co-culture model, St-T1b cells were
788 embedded in growth factor-reduced Matrigel, with a single layer of EECs seeded on top. **[B]** In-cell
789 western® analysis was conducted to quantify epithelial integrin αVβ3 expression with or without
790 treatment with medroxyprogesterone acetate, cyclic adenosine monophosphate and 17β-estradiol,
791 on days 4, 6 and 8. Results are mean ± SEM of eight separate experiments. $^{**}P < 0.05$; $^{***}P < 0.0001$;
792 one-way ANOVA with Tukey's multiple comparison post-test analysis.

Table 1

Timeline	Hormonal stimuli added with MM1	Control (unstimulated cells)
Day 0	0.25 μM MPA + 0.25 mM 8-Br-cAMP + 1 nM E2	MM1 + 0.001% EtOH
Days 2, 4 and 6	1 μM MPA + 0.5 mM 8-Br-cAMP + 10 nM E2	MM1 + 0.001% EtOH

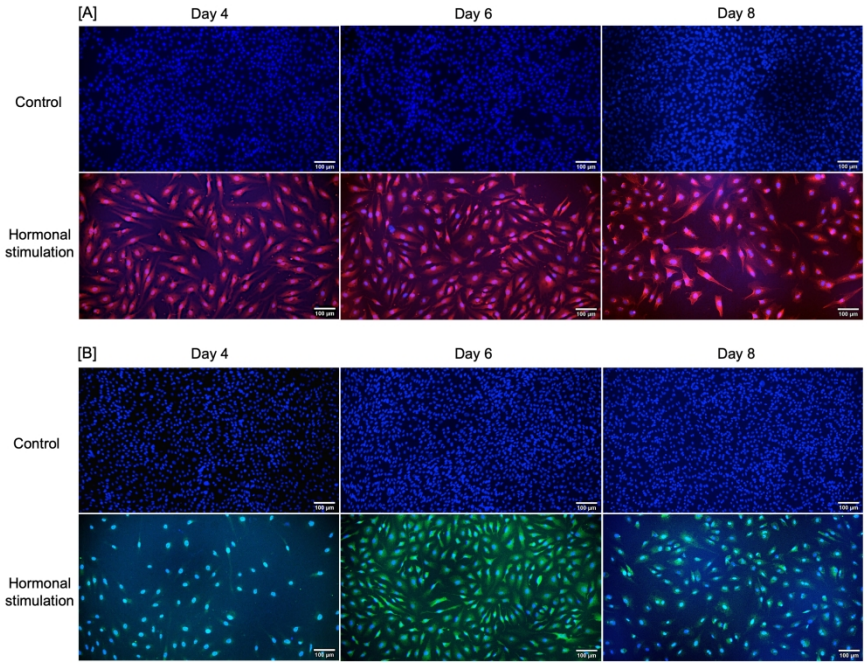

Figure 1: Hormonal stimulation of St-T1b cells with medroxyprogesterone acetate, 8-bromoadenosine 3',5'-cyclic adenosine monophosphate and 17β-estradiol. Decidualisation confirmed by immunohistochemical detection of [A] IGFBP-1 expression, and [B] the transient upregulation of ST2L expression. Scale bars = 100 μm.

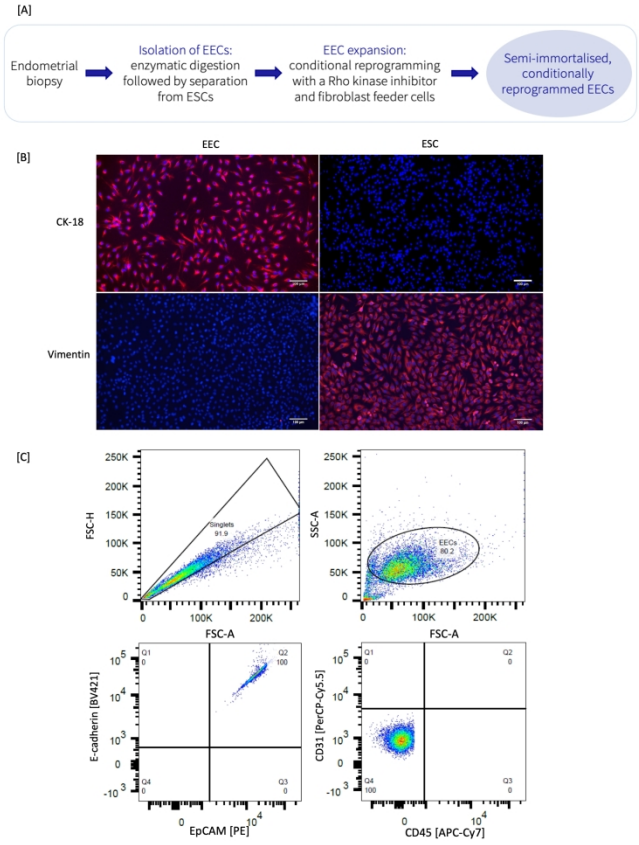

Figure 2: Endometrial epithelial cells (EECs) were isolated from endometrial biopsies and expanded in vitro by conditional reprogramming. Confirmation of epithelial phenotype by [B] immunocytochemical analyses demonstrated cytokeratin-18 (CK18) expression and negative vimentin staining in EECs, with endometrial stromal cells (ESCs) serving as negative and positive controls, respectively. Scale bars = 100 µm. [C] Flow cytometric analyses demonstrated EpCAM and E-cadherin expression by EECs, but no CD31 and CD45 expression.

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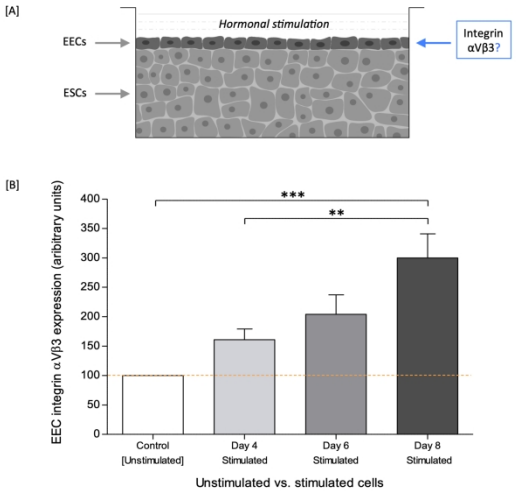

Figure 3. [A] To construct a 3D endometrial organotypic co-culture model, St-T1b cells were embedded in growth factor-reduced Matrigel, with a single layer of EECs seeded on top. [B] In-cell western analysis was conducted to quantify epithelial integrin αVβ3 expression with or without treatment with medroxyprogesterone acetate, cyclic adenosine monophosphate and 17β-estradiol, on days 4, 6 and 8. Results are mean ± SEM of eight separate experiments. **P <0.05; ***P < 0.0001; one-way ANOVA with Tukey's multiple comparison post-test analysis.

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Supplementary table and figure legends

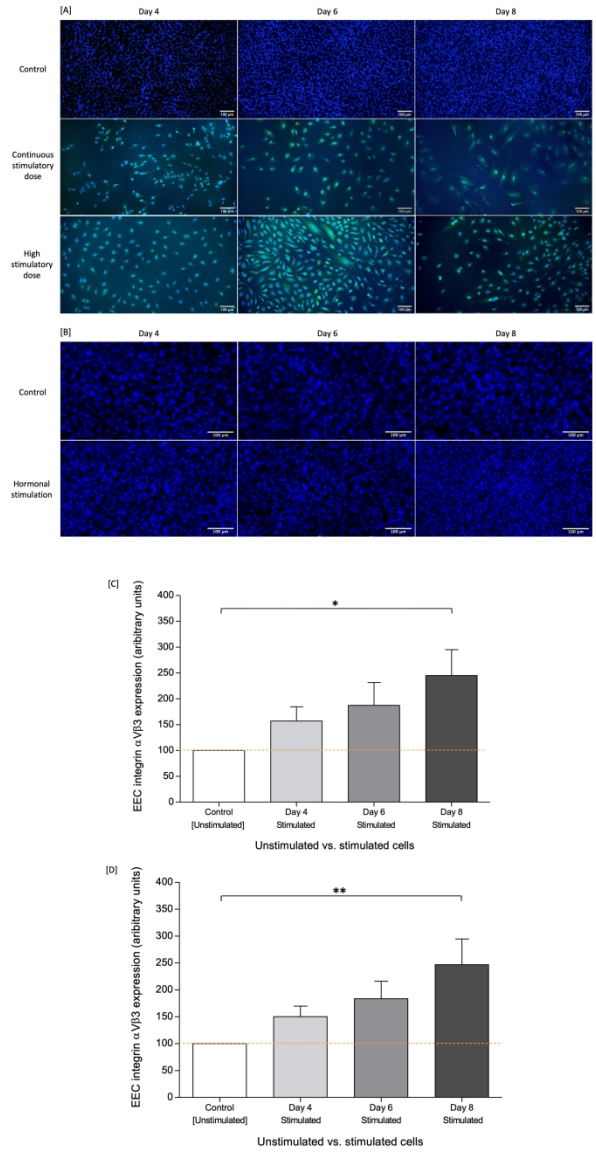
Supplementary table 1: ***Continuous stimulatory dose:*** St-T1b cells were stimulated with minimal medium 1 (MM1) containing medroxyprogesterone acetate (MPA), 8-bromoadenosine 3',5'-cyclic adenosine monophosphate (8-Br-cAMP) and 17β-estradiol (E2), or MM1 with 0.001% ethanol (EtOH), every 48 hours, and monitored over 8 days.

Supplementary table 2: ***High stimulatory dose:*** to produce a decidual response to doses exceeding physiological levels, St-T1b cells were stimulated with minimal medium 1 (MM1) containing increasing concentrations of medroxyprogesterone acetate (MPA), 8-bromoadenosine 3',5'-cyclic adenosine monophosphate (8-Br-cAMP) and 17β-estradiol (E2) , or MM1 with 0.001% ethanol (EtOH), every 48 hours, and monitored over 8 days.

Supplementary figure 1: **[A]** ST2L expression by St-T1b cells following stimulation with continuous stimulatory doses (Supplementary table 1) or high stimulatory doses (Supplementary table 2) of medroxyprogesterone acetate, 8-bromoadenosine 3',5'-cyclic adenosine monophosphate and 17β-estradiol. Scale bars = 100 μm**. [B]** Integrin αVβ3 expression by endometrial epithelial cells (EECs) following stimulation with increasing physiological stimulatory hormonal doses (Table 1). Scale bars = 100 μm. St-T1b cells were embedded in growth factor-reduced Matrigel, with a single layer of EECs seeded on top, to produce a 3D endometrial organotypic co-culture model. In-cell western® analysis was conducted to quantify EEC integrin αVβ3 expression upon treatment of the 3D co-culture system with **[C]** continuous stimulatory doses (Supplementary table 1) or **[D]** high stimulatory doses (Supplementary table 2). Results are mean ± SEM of at least eight separate experiments. **P* <0.05; ***P* < 0.001; one-way ANOVA with Tukey's multiple comparison post-test analysis.

Supplementary table 3: Representative in-cell western (ICW) data (arbitrary units indicating epithelial integrin αVβ3 expression) for Figure 3B (increasing physiological stimulatory hormonal doses), Supplementary figure 1A (continuous stimulatory dose) and Supplementary figure 1B (high, increasing stimulatory doses).

Supplementary table 4: Pooled (n = 8) raw in-cell western data (arbitrary units indicating epithelial integrin αVβ3 expression) for Figure 3B.



Supplementary Figure 1

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Supplementary table 1

Timeline	Hormonal stimuli added with MM1	Control (unstimulated cells)
Days 0, 2, 4 and 6	1 μM MPA + 0.5 mM 8-Br-cAMP + 10 nM E2	MM1 + 0.001% EtOH

Supplementary table 2

Timeline	Hormonal stimuli added with MM1	Control (unstimulated cells)
Day 0	0.25 μM MPA + 0.25 mM 8-Br-cAMP + 1 nM E2	MM1 + 0.001% EtOH
Day 2	1 μM MPA + 0.5 mM 8-Br-cAMP + 5 nM E2	MM1 + 0.001% EtOH
Days 4 and 6	1.5 μM MPA + 0.75 mM 8-Br-cAMP + 10 nM E2	MM1 + 0.001% EtOH

Supplementary table 3

ICW data for Figure 3B			
Control (unstimulated)	Day 4 (stimulated)	Day 6 (stimulated)	Day 8 (stimulated)
100	149.0756	154.6218	300.1681
100	112.471	88.8437	158.6287
100	267.8411	222.1139	447.6012
100	135.1058	138.3982	149.2945
100	140.3425	141.0379	227.453
100	129.3527	205.7315	296.1996
100	210.4804	356.5579	418.6499
100	142.8542	324.3868	401.865
100	**152.6217733**	**194.3709778**	**277.0797922**
ICW data for Supplementary figure 1B			
Control (unstimulated)	Day 4 (stimulated)	Day 6 (stimulated)	Day 8 (stimulated)
100	75.19325	239.1597	355.6303
100	118.629	148.5476	190.55
100	74.74048	97.92388	98.05363
100	315.8171	88.8437	158.6287
100	135.5762	171.2046	212.3987

100	163.675	126.7834	181.522
100	196.3695	152.1088	217.7816
100	175.9984	473.9915	545.8747
100	**156.9998663**	**187.3203975**	**245.0549538**
ICW data for Supplementary figure 1C			
Control (unstimulated)	Day 4 (stimulated)	Day 6 (stimulated)	Day 8 (stimulated)
100	211.0924	187.0588	267.2269
100	91.67312	134.3726	80.86754
100	71.88581	143.8581	148.7024
100	208.3208	414.9925	514.3928
100	122.5634	108.7667	118.1735
100	133.3119	133.7754	240.9735
100	117.1173	117.3026	180.7508
100	153.6793	153.9226	237.1782
100	242.0323	256.5242	433.3141
100	211.0924	187.0588	267.2269
100	**150.1862589**	**183.3970556**	**246.8421933**

Supplementary table 4

	Day 4	Day 6	Day 8
Control (unstimulated)	**17.348**	**19.104**	**18.711**
Hormonal stimulation (increasing physiological stimulatory doses)	**26.741**	**34.389**	**46.93**